ONE-CARBON (C-1) METABOLISM IN RESPONSE TO BIOTIC AND ABIOTIC STRESSES

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ABSTRACT

In plants, the generation and supply of methyl units is important in one-carbon (C-1) metabolism, which is essential to all organisms. I have identified a series of cDNA sequences encoding N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR), cobalamin-independent methionine synthase (Met Syn), *S*-adenosylmethionine synthetase (isoform I, AdoMet Syn2661 and isoform II, AdoMet Syn605), *S*-adenosylmethionine decarboxylase (SAMDC), serine hydroxymethyltransferase (SHMT) and N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFC/THFD) in the pathways of generation and supply of methyl units. These are from a cDNA library of mRNA from a susceptible wheat (*Triticum monococcum*) (Tm) line 441 epidermis, 24 h after inoculation with powdery mildew fungus (*Blumeria graminis* f. sp. *tritici*) (*Bgt*). Phylogenetic tree cluster analysis and subcellular localization prediction by TargetP revealed that MTHFR, Met Syn, AdoMet Syn605, AdoMet Syn2661, SAMDC, and THFC/THFD may be localized in cytosol; SHMT may be localized in mitochondria. Northern blot analysis indicated that expression of *MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605* and *SAMDC* genes was up-regulated by powdery mildew infection, abiotic stresses and treatments with stress signal molecules; expression of *SHMT and THFC/THFD* was either constitutive or down-regulated. These results suggest a close metabolic link between various stresses and the pathways of generation and supply of methyl units in this wheat.

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ABA	abscisic acid
ADC	arginine decarboxylase
AdoHcy	*S*-adenosylhomocysteine
AdoHcy Hy	*S*-adenosylhomocysteine hydrolase
AdoMet	*S*-adenosylmethionine
AdoMet Hy	*S*-adenosylmethionine hydrolase
AdoMet Syn	*S*-adenosylmethionine synthetase
AGT	appressorial germ tube
AHAT	alanine-hydroxypyruvate aminotransferase
AOA	α-aminooxyacetate
AOPP	α-aminooxy-β-phenylpropionic acid
APX	ascorbate peroxidase
ATI	alfalfa trypsin inhibitor
Bgt	*Blumeria graminis* f. sp. *tritici*
BLAST	basic local alignment search tool
bp	base pair
C-1	one-carbon
CAD	cinnamyl alcohol dehydrogenase
CCoAOMT	*S*-adenosylmethionine: trans-caffeoyl-coenzyme A 3-*O*-methyl-transferase
CK	control
COMT	*S*-adenosylmethionine: caffeic acid 3-*O*-methyltransferase
EST	expressed sequence tag
GA	gibberellic acid
GDC	glycine decarboxylase complex
GDH	glycerate dehydrogenase
GHAT	glycine-hydroxypyruvate aminotransferase
GPX	glutathione peroxidase
GST	glutathione *S*-transferase
gus	glucuronidase
h	hour
H_2O_2	hydrogen peroxide
hpi	hours post inoculation
HR	hypersensitive reaction
JA	jasmonic acid
kB	kilobase
kDa	kilodalton
MeJA	methyl jasmonate
Met Syn	cobalamin-independent methionine synthase
MTHFR	N^5, N^{10}-methylenetetrahydrofolate reductase
N^5, N^{10}-methylene-THF	N^5, N^{10}-methylenetetrahydrofolate
N^5-methyl-THF	N^5-methyltetrahydrofolate
NCBI	national center for biotechnology information

NO	nitric oxide
O_2^-	superoxide radical
O_2	oxygen
O_3	activated oxygen
ODC	ornithine decarboxylase
$OH^·$	hydroxyl radical
OH-PAS	*N*-*O*-hydroxyphenyl- sulfinamoyl-tertiobutyl acetate
OMT	*O*-methyltransferase
OPH	*O*-phosphohomoserine
ORF	open reading frame
PAL	phenylalanine ammonia-lyase
PEST	Pro, Glu, Ser, Thr
PGDH	3-phosphoglycerate dehydrogenase
PGP	3-phosphoglycerate phosphatase
PGT	primary germ tube
PHGPX	phospholipids hydroperoxide glutathione peroxidase
pI	isoelectric point
PR	pathogenesis-related
PSAT	3-phosphoserine aminotransferase
PSP	3-phosphoserine phosphatase
SA	salicylic acid
SAMDC	*S*-adenosylmethionine decarboxylase
SAR	systemic acquired resistance
SHMT	serine hydroxymethyltransferase
SOD	superoxide dismutase
THF	tetrahydrofolate
THFC/THFD	N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase
Tm	*Triticum monococcum*
TMV	tobacco mosaic virus
uORF	upstream open reading frame
UTR	untranslated region
WALI	aluminum-induced genes
WIP	wound-induced protein

1. INTRODUCTION

Plants not only defend themselves against attack from biotic stressors such as bacteria, viruses, fungi and other plants, but also are constantly exposed to changes in their environment. They also perceive a variety of abiotic stresses including wounding, drought, cold and salinity, as well as perceive stress signal molecules. Both kinds of stresses, biotic and abiotic, can greatly lower crop yields and quality. Research has shown that if crop yields can be assumed to represent plant growth under ideal conditions, then the losses associated with biotic and abiotic stresses can reduce average productivity by 65% to 87% (Bray et al., 2000).

Many functionally identified elicitor-stimulated genes are related to secondary metabolism, such as phenylalanine ammonia-lyase (*PAL*) (Lois et al., 1989), peroxidase and tyrosine-rich hydroxyproline-rich glycoprotein (Kawalleck et al., 1992). The discovery by Kawalleck et al. (1992) of *S*-adenosylmethionine synthetase (AdoMet Syn) and *S*-adenosylhomocysteine hydrolase (AdoHcy Hy) mRNA induced by fungal elicitor in cultured parsley cells and leaves represented a new find. Induced *AdoMet Syn* gene expression in response to an elicitor from baker's yeast was also found in alfalfa cells (Gowri et al., 1991). These discoveries led me to investigate whether expression of the genes involved in C-1 metabolism is also up-regulated in response to biotic stress, as well as to a series of abiotic stresses.

One-carbon (C-1) metabolism is essential to all organisms. In plants, it supplies the C-1 units needed to synthesize proteins, nucleic acids, pantothenate, and many methylated molecules (Cossins and Chen, 1997). There are many enzymes and pathways involved in C-1 metabolism, among which the C1-THF pathways and the activated methyl cycle are particularly important. These pathways are interrelated (Fig. 2.1).

Some research has been done on genes encoding enzymes involved in the C-1 metabolic response to stresses, such as *AdoMet Syn* and *AdoHcy Hy* in response to fungal elicitor in parsley cells (Kawalleck et al., 1992); *AdoMet Syn* to an elicitor from baker's yeast in alfalfa cells (Gowri et al., 1991); *AdoMet Syn* to salt and ABA treatments in tomato (Espartero et al., 1994); *S*-adenosylmethionine decarboxylase (*SAMDC*) to salt and drought treatments in wheat and rice (Li and Chen, 2000a, 2000b); cobalamin-independent methionine synthase (*Met Syn*) to alkaline stress in rice (Xie et al., 2002). But this is the first time an attempt has been made to define and relate the expression of a full set of genes encoding enzymes involved in the pathways of generation and supply of methyl units (Fig. 2.1) to a wide array of biotic and abiotic stresses. Elucidating the relationship between C-1 metabolism and biotic and abiotic stresses and knowledge of the gene expression pattern in these pathways in response to stresses will improve our understanding of C-1 metabolism and of the mechanism of plant resistance to stresses.

Engineered modification of C-1 fluxes in plants has been found in the area of AdoMet metabolism (Boerjan et al., 1994; Good et al., 1994; Tanaka et al., 1997). In order to lower AdoMet levels, the gene from bacteriophage T3 that encodes the enzyme *S*-adenosylmethionine hydrolase (AdoMet Hy) was utilized to generate transgenic tomato plants. The transformants produced less ethylene, which suggests the AdoMet pool was

depleted (Good et al., 1994). Tanaka et al. (1997) found that tobacco plants were morphologically abnormal and showed hypomethylation of DNA when AdoHcy Hy activity was lowered by antisense RNA expression. Such engineering research, together with studies of how C-1 gene expression patterns changed in the engineered plants, will provide new light on how C-1 fluxes are controlled in plants.

In pursuing an understanding of the relationship between the enzymes involved in C-1 metabolism and the stresses imposed on plants, I undertook to seek answers to these questions:

1. Which genes coding for enzymes in C-1 metabolism are up-regulated?
2. What is the difference between responses provoked by the invasion of a pathogenic organism and those provoked by mechanical wounding or by the stresses of low temperature, high salinity, or desiccation?
3. Which genes in the pathways are regulated coordinately in response to stresses?
4. To which subcellular compartments are the genes involved in the C-1 metabolism targetted?

2. LITERATURE REVIEW

2.1 One-carbon (C-1) metabolism in response to biotic and abiotic stresses

There are many enzymes and pathways involved in C-1 metabolism. Of particular importance are the pathways for generation and supply of methyl units, which include the activated methyl cycle and folate-mediated C-1 reactions, as demonstrated in Fig. 2.1.

Some important enzymes are involved in these pathways, including: N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR), cobalamin-independent methionine synthase (Met Syn), *S*-adenosylmethionine synthetase (AdoMet Syn), *S*-adenosylmethionine decarboxylase (SAMDC), serine hydroxymethyltransferase (SHMT) and N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFC/THFD) (Fig. 2.1).

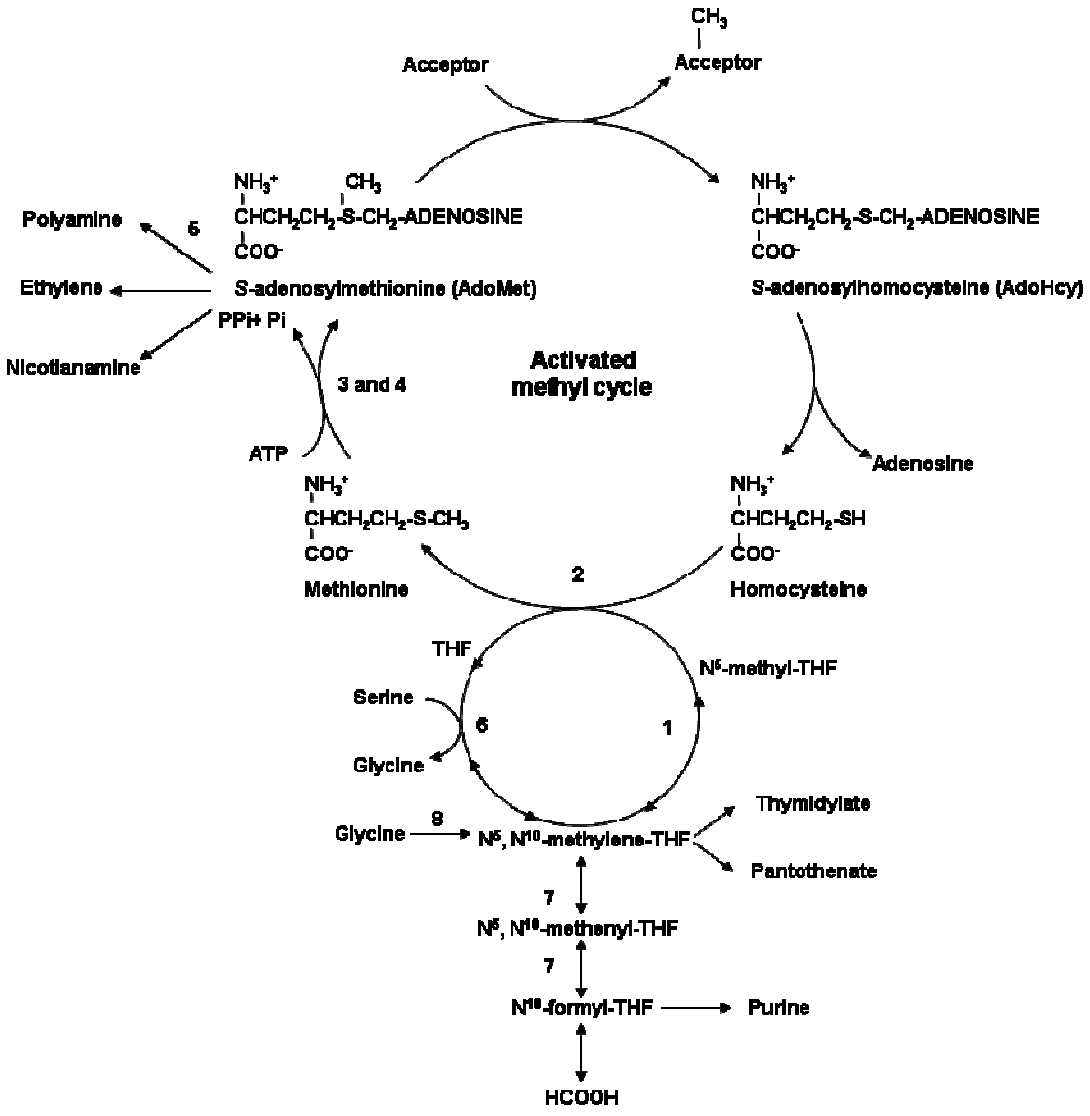

Fig. 2.1 The pathways of generation and supply of methyl units.

1. N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR)
2. cobalamin-independent methionine synthase (Met Syn)
3. *S*-adenosylmethionine synthetase2661 (AdoMet Syn2661)
4. *S*-adenosylmethionine synthetase605 (AdoMet Syn605)
5. *S*-adenosylmethionine decarboxylase (SAMDC)
6. serine hydroxymethyltransferase (SHMT)
7. N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFC/THFD)
8. glycine decarboxylase complex (GDC)

2.1.1 N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR)

MTHFR plays a major role in the metabolism of folates. It catalyzes the reduction of N^5, N^{10}-methylenetetrahydrofolate (N^5, N^{10}-methylene-THF) to N^5-methyltetrahydrofolate (N^5-methyl-THF), which serves as a methyl donor for the methylation of homocysteine to form methionine, the terminal step in methionine synthesis. Thus, MTHFR is critical in keeping low homocysteine levels. Recently, an elevated homocysteine level due to deficiency in MTHFR has been implicated as a risk factor for neural tube defects (Rosenblatt, 1995) and vascular diseases (Frosst et al., 1995). Genes of MTHFR in pig liver (Daubner and Matthews, 1982), human and mouse (Goyette et al., 1998), *Escherichia coli* (Sheppard et al., 1999) and *Saccharomyces cerevisiae* (Raymond et al., 1999) have been characterized. Roje et al. (1999) isolated and characterized cDNAs encoding MTHFR from maize and *Arabidopsis*. Deduced amino acid sequences showed that the molecular mass of the plant polypeptides is 66 kilodalton (KDa), that they lacked obvious targeting sequences and that they have similar domains to other eukaryotic MTHFRs. All known eukaryotic MTHFRs have a N-terminal domain which contains the catalytic site and a C-terminal domain that is implicated in regulating enzyme activity (Matthews et al., 1984; Goyette et al., 1994). The mammalian MTHFR C-terminal domain contains the *S*-adenosylmethionine (AdoMet)-binding site and has been shown to be inhibited by AdoMet. This sensitivity is considered important to prevent N^5, N^{10}-methylene-THF depletion (Appling, 1991; Bagley and Selhub, 1998).

In contrast to the detailed information about mammalian MTHFRs, there is little research on plant MTHFRs, thus making it the least understood enzyme of folate-

mediated C-1 metabolism in plants (Roje et al., 1999). Plant MTHFRs are not inhibited by AdoMet and prefer NADH to NADPH; the function of the C-terminal domain of plant MTHFRs is still unknown (Roje et al., 1999).

2.1.2 Cobalamin-independent methionine synthase (Met Syn)

In higher plants, methionine is synthesized from cysteine and *O*-phosphohomoserine (OPH), a series of reactions catalyzed by cystathionine *γ*-synthase to form cystathionine, followed by cystathionine *β*-lyase to produce homocysteine and finally, a step involving the transfer a methyl group from N^5-methyl-THF to homocysteine, a reaction catalyzed by Met Syn. Met Syn not only catalyzes the last reaction in *de novo* methionine synthesis but also regenerates the methyl group of AdoMet from *S*-adenosylhomocysteine (AdoHcy) after methylation reactions. AdoMet is a compound that serves as the methyl donor for a large number of biological reactions such as syntheses of choline, polyamine and ethylene, and DNA methylation (Ravanel et al., 1998). AdoMet is used for transmethylation reactions in which the methyl group of methionine is transferred to an acceptor, leaving AdoHcy as a by-product. AdoHcy is then converted to homocysteine, a reaction catalyzed by AdoHcy hydrolase. Methionine is then regenerated through methylation of homocysteine.

There are two types of Met Syn. One, cobalamin-dependent, which contains a cobalamin cofactor and uses either mono- or polyglutamylated N^5-methyl-THF (5-$CH_3H_4PteGlu_n$, $n = 1$ or $n \geq 3$) as methyl donor, has been found predominantly in higher eukaryotes (Chen et al., 1997; Yamada et al., 1998). The second type is cobalamin-independent and uses only the polyglutamylated form of N^5-methyl-THF (5-$CH_3H_4PteGlu_n$, $n \geq 3$) as methyl donor. The latter enzyme has been purified and cloned

from plants and bacteria (Eichel et al., 1995; Moestrup et al., 1996; Kurvari et al., 1995; González et al., 1992; Ravanel et al., 1998).

Eichel et al. (1995) first reported cloning the plant cobalamin-independent *Met Syn* in *Catharanthus roseus*. This cDNA was found to encode a slightly acidic protein of 85 KDa; immunoblots showed the protein to be localized in the cytosol. Shown also was the stress-induced transient increase of the transcription level of the gene coding for this enzyme. Nutritional deprivation of nitrogenous compounds and 2, 4-dichlorophenoxy-acetic acid in the medium of the *Catharanthus roseus* cell culture led to a strong, transient transcription activation but no significant increase in the protein level. A similar result was observed in the case of *AdoMet Syn* and *AdoHcy Hy* (Eichel et al., 1995). Although the transcription level was up-regulated, the protein content remained constant throughout the experimental period, perhaps because the regulation of protein expression was under independent control.

Zeh et al. (2002) cloned a cDNA encoding a cobalamin-independent Met Syn from potato. Results of northern blots revealed that the expression of the potato *Met Syn* gene was differentially regulated in a tissue-specific manner. Feeding experiments of detached leaves showed that this gene expression was regulated at the level of transcription but not at the level of protein, which was similar to *Catharanthus roseus* (Eichel et al., 1995).

The gene for a cobalamin-independent Met Syn cloned from rice was tested for responsiveness to alkaline stress (Xie et al., 2002). Results indicated that the transcription level was enhanced at 12 h and 24 h after treatment and then was reduced sharply by 48 h, suggesting that this gene is involved in adaptation to alkaline stress.

2.1.3 S-adenosylmethionine synthetase (AdoMet Syn)

AdoMet Syn catalyzes the formation of AdoMet from Met and ATP. AdoMet is a universal methyl group donor in transmethylation reactions involving many kinds of acceptors such as nucleic acids, lipids, proteins, cell-wall pectic polysaccharides and phenylpropanoid derivatives (Van Doorsselaere et al., 1993). It also serves as a propylamine group donor in the biosynthesis of polyamines, which play a crucial role in plant growth and development (Peleman et al., 1989b; Lamblin et al., 2001). In plants, AdoMet has been studied mainly in connection with the biosynthesis of various phenylpropanoid derivatives and as a precursor in the biosynthesis of ethylene (Yang and Hoffman, 1984).

So far, *AdoMet Syn* genes in plants have been isolated from *Arabidopsis thaliana* (Peleman et al., 1989a, 1989b), carnation (Larsen and Woodson, 1991), parsley (Kawalleck et al., 1992), poplar (Van Doorsselaere et al., 1993), rice (Van Breusegem et al., 1994), tomato (Espartero et al., 1994), kiwifruit (Whittaker et al., 1995), petunia (Izhaki et al., 1995) and pea (Gómez-Gómez and Carrasco, 1996).

Arabidopsis thaliana contains two *AdoMet Syn* genes, *AdoMet Syn1* and *AdoMet Syn2*. Both genes are expressed primarily in vascular tissues (Peleman et al., 1989a, 1989b). These two *AdoMet Syn* genes show similar expression patterns in various organs; transcription levels are 10 to 20 times higher in roots and stems than in leaves and flowers (Peleman et al., 1989b). In rice, a similar level of *AdoMet Syn* gene expression was found in roots, leaves and cell suspension (Van Breusegem et al., 1994), which is different from the situation in *Arabidopsis thaliana*. The uniform expression of the rice

gene probably reflected the high level of lignified tissue in rice leaves compared to dicotyledonous plants (Van Breusegem et al., 1994).

Gómez-Gómez and Carrasco (1998) demonstrated that different *AdoMet Syn* genes have differential expression levels during pea development. There are two *AdoMet Syns* in pea. Expression of *AdoMet Syn1* was specifically up-regulated after pollination, whereas *AdoMet Syn2* was expressed constitutively. Both *AdoMet Syn1* and *AdoMet Syn2* showed increased expression in senescing ovaries. Ethylene treatment of unpollinated ovaries led to an increase in *AdoMet Syn1* mRNA level, but *AdoMet Syn2* expression remained unchanged. In contrast, the *AdoMet Syn* mRNA in carnation flowers decreased during senescence (Woodson et al., 1992). Similar results were shown in petunia flowers where, upon flower opening, the level of *AdoMet Syn* mRNA began declining (Izhaki et al., 1995).

Recent studies have indicated that *AdoMet Syn* genes are expressed in both an organ-specific and gene-specific manner in response to NaCl stress in *Catharanthus roseus* (Schröder et al., 1997) and tomato (Espartero et al., 1994). There are three *AdoMet Syns* in *Catharanthus roseus.* These three closely-related AdoMet Syn isoenzymes from *Catharanthus roseus* are differentially expressed in cell cultures during growth of the culture and after application of various stresses, such as increased NaCl, elicitor and nutritional down-shift (Schröder et al., 1997). There are three *AdoMet Syns* in tomato. Northern blot analysis also demonstrated a differential response of 3 *AdoMet Syn* genes to NaCl, mannitol and ABA treatments (Espartero et al., 1994). *AdoMet Syn1* and *AdoMet Syn3* mRNAs accumulated in roots in response to the three-stress treatments; *AdoMet Syn1* mRNA also accumulated in leaves. The response of the *AdoMet Syn* gene

to wounding showed that only small changes in steady-state levels of *AdoMet Syn* mRNAs occurred except for the significant *AdoMet Syn3* mRNA increase in roots (Espartero et al., 1994). Lee et al (1997) further examined the effects of NaCl stress on rice *AdoMet Syn* gene expression and found that the rice *AdoMet Syn* genes were differentially regulated in a tissue-specific manner while being coordinately expressed during growth of the rice cell culture. There are two *AdoMet Syns* in rice. *AdoMet Syn1* mRNA was not increased after salt stress while accumulation of *AdoMet Syn2* transcript was clearly induced by NaCl treatment. These results suggested that *AdoMet Syn2* plays a role in the response of the cells to salt stress. Rice plants removed from their growth medium for 15-180 min became drought stressed, which led to 2- to 4-fold increase in the *AdoMet Syn* gene transcript level (Van Breusegem et al., 1994). Recently, some investigations have indicated that *AdoMet Syn* gene expression is induced by the phytohormone gibberellic acid (GA) in wheat aleurones (Mathur et al., 1992), dwarf pea epicotyls (Mathur and Sachar, 1991) and petunia corollas and stems (Izhaki et al., 1996).

Studies have shown a close metabolic link between pathogen defense and an increased turnover of activated methyl groups. Kawalleck et al. (1992) found that elicitor at 50 μg/ml derived from the fungus *Phytophthora megasperma* f. sp. *glycinea* strongly induced *AdoMet Syn* and *AdoHcy Hy* mRNA accumulation in cultured parsley cells and intact leaves, which led to marked increases in AdoHcy Hy enzyme activity. Gowri et al. (1991) investigated the induction of *AdoMet Syn* and *S*-adenosylmethionine: caffeic acid 3-*O*-methyltransferase (*COMT*) transcript level in alfalfa cell suspension cultures in response to an elicitor from bakers' yeast. Results showed that expression of *AdoMet Syn* was co-induced with *COMT* transcript in elicitor-treated cells. Northern blot

analysis revealed that *COMT* transcripts are most abundant in roots and stems; similar results were found for *AdoMet Syn*. Busam et al (1997) further showed that treatment of the cultured *Vitis vinifera* L. cells with fungal elicitor raised the *S*-adenosylmethionine: trans-caffeoyl-coenzyme A 3-*O*-methyl-transferase (*CCoAOMT*) transcript abundance, suggesting a role for *CCoAOMT* in the disease-resistance response. Jaeck et al. (1996) examined expression of class I *O*-methyltransferase (OMT I) genes in healthy and tobacco mosaic virus (TMV)-infected tobacco. Results indicated that OMT I genes were particularly expressed in vascular cells and epidermis in the stem, petiole and root. In healthy leaves *OMT I* mRNA was detected only in vascular strands, whereas in TMV-infected tobacco leaves a strong accumulation of labeling was also localized in the upper and lower epidermis. Giampiero et al. (1993) also found that treatment of *Arabidopsis* cells with fungal elicitor leads to rapid accumulation of certain mRNAs, including *AdoMet Syn* and *AdoHcy Hy*, with time courses comparable to those observed for their counterparts in parsley. These results suggested a close metabolic link between pathogen defense and an increased turnover of activated methyl groups.

2.1.4 S-adenosylmethionine decarboxylase (SAMDC)

SAMDC is an important enzyme in polyamine biosynthesis (Pegg, 1986); it was possible the rate-limiting enzyme in the biosynthesis of polyamines (Tabor and Tabor, 1984). SAMDC catalyzes the production of decarboxylated AdoMet, which is the methyl group donor for most cellular methylation reactions and the aminopropyl moiety donor for spermidine and spermine biosynthesis (Walden et al., 1997). The gene encoding this enzyme in plants has been cloned from potato (Mad Arif et al., 1994), *Catharanthus roseus* (Schröder and Schröder, 1995), spinach (Bolle et al., 1995), barley

(Dresselhaus et al., 1996), carnation (Lee et al., 1997), rice (Li and Chen, 1999), wheat (Li and Chen, 2000a), *Arabidopsis thaliana* (Franceschetti et al., 2001) and soybean (Tian et al., 2004).

Polyamines are essential for growth and development. Polyamines elicit diverse physiological responses including cell division, tuber formation, floral and fruit development, root initiation, embryogenesis and stresses (Evans and Malmberg, 1989; Walden et al., 1997).

Li and Chen (2000a) isolated the full-length cDNA for SAMDC from wheat (*Triticum aestivum* L.) and observed that salinity, drought and exogenous ABA all induced its expression. Northern hybridization showed that the transcript of *SAMDC* was accumulated steadily and reached a peak at 24 h after the start of salt stress; application of exogenous ABA resulted in the elevation of the transcript after 12 hr; only slight elevation occurred in wheat seedlings after drought stress. Li and Chen (2000b) also found that *SAMDC* transcript level was induced by salinity, drought and exogenous ABA in rice (*Oryza sativa* L.). There was a different pattern of transcription level accumulation in salt-tolerant rice variety Lansheng and salt-sensitive variety 77-170 in response to salinity stress. It was observed that the level of *SAMDC* transcript occurred earlier in Lansheng than in 77-170 and the *SAMDC* transcript was detected in Lansheng under low salt conditions and in 77-170 under high salt conditions (Li and Chen, 2000b). Tian et al. (2004) cloned and characterized a *SAMDC* gene from soybean. Northern blot analysis demonstrated that the *SAMDC* gene expression was induced by drought, salt and cold.

Mad Arif et al. (1994) characterized a potato *SAMDC* with a molecular mass of 39 KDa. Northern blot analysis showed that the *SAMDC* was highly expressed in actively dividing

and young tissues but much less so in non-dividing and mature tissues of both reproductive and vegetative organs. Marco and Carrasco (2002) further showed a differential expression of the *SAMDC* of pea in reproductive and vegetative tissues. *SAMDC* mRNA levels were higher in stems and roots than in leaves.

Recent studies found that treatment with jasmonates can increase the accumulation of polyamines (Lee et al., 1997; Biondi et al., 2000; Mader, 1999). Biondi et al. (2001) indicated that both transcription and protein levels of *SAMDC* were up-regulated in methyl jasmonate (MeJA)-treated thin layers of tobacco tissues. Treatment of barley seedlings with MeJA led to the accumulation of polyamines, as well as increased activities of SAMDC (Walters et al., 2002).

2.1.5 Serine hydroxymethyltransferase (SHMT)

The first enzyme in the pathway for the interconversion of C-1 compounds is serine hydroxymethyltransferase (SHMT) which catalyzes the reversible conversion of serine and tetrahydrofolate (THF) to glycine and N^5**,** N^{10}-methylene-THF (Schirch, 1982). In addition to its reduction to N^5-methyl-THF, the N^5**,** N^{10}-methylene-THF is converted via a series of reactions to produce thymidylate, purines etc. The N^5-methyl-THF then serves as a methyl donor for the methylation of homocysteine to form methionine whose central role is in methyltransfer reactions via AdoMet to form important biological compounds, such as lignin, pectin and plastoquinones (Dodd and Cossins, 1969). Thus, a systematic investigation of the regulation of the enzymes of the C1-THF pathway is essential for understanding the mechanism of the biosynthesis pathway.

Serine can be formed by several pathways, the principal one being associated with photorespiratory glycine metabolism through the THF-mediated conversion of glycine

to serine by glycine decarboxylase complex (GDC) and SHMT; the second is the THF-mediated pathway via the C1-THF synthase (THFC/THFD and N^{10}-formyl-THF synthetase) and SHMT using formate as the C-1 source (Prabhu et al., 1996; King, 2002); the third and fourth are the phosphorylated and non-phosphorylated pathways (Ho and Saito, 2001; Kleczkowski and Givan, 1988; Bourguignon et al., 1998).

Serine and glycine are both potential sources of C-1 units in plants. They are interconvertible via SHMT. GDC oxidizes glycine to CO_2, NH_3 and N^5, N^{10}-methylene-THF (reaction 1 and Fig. 2.2). The N^5, N^{10}-methylene-THF reacts with a second molecule of glycine, catalyzed by SHMT, to produce serine (Oliver, 1994; Prabhu et al., 1996; reaction 2 and Fig. 2.2).

$$\text{Glycine} + NAD^+ + \text{THF} \xrightarrow{\text{GDC}} N^5, N^{10}\text{-methylene-THF} + CO_2 + NH_3 + \text{NADH} \quad (1)$$

$$\text{Glycine} + N^5, N^{10}\text{-methylene-THF} + H_2O \xleftrightarrow{\text{SHMT}} \text{Serine} + \text{THF} \quad (2)$$

Formate is also a C-1 source for the generation of N^5, N^{10}-methylene-THF. Three sequential reactions carry out this conversion: N^{10}-formyl-THF synthetase (reaction 3 and Fig. 2.2), THFC (reaction 4 and Fig. 2.2) and THFD (reaction 5 and Fig. 2.2), collectively termed C1-THF synthase (Prabhu et al., 1996). All three reactions are reversible:

$$\text{Formate} + \text{THF} + \text{ATP} \xleftrightarrow{N^{10}\text{-formyl-THF synthetase}} N^{10}\text{-formyl-THF} + \text{ADP} + \text{Phosphate} \quad (3)$$

$$N^{10}\text{-formyl-THF} + H^+ \xleftrightarrow{\text{THFC}} N^5, N^{10}\text{-methenyl-THF} + H_2O \quad (4)$$

$$N^5, N^{10}\text{-methenyl-THF} + \text{NAD(P)H} \xleftrightarrow{\text{THFD}} N^5, N^{10}\text{-methylene-THF} + \text{NAD(P)}^+ \quad (5)$$

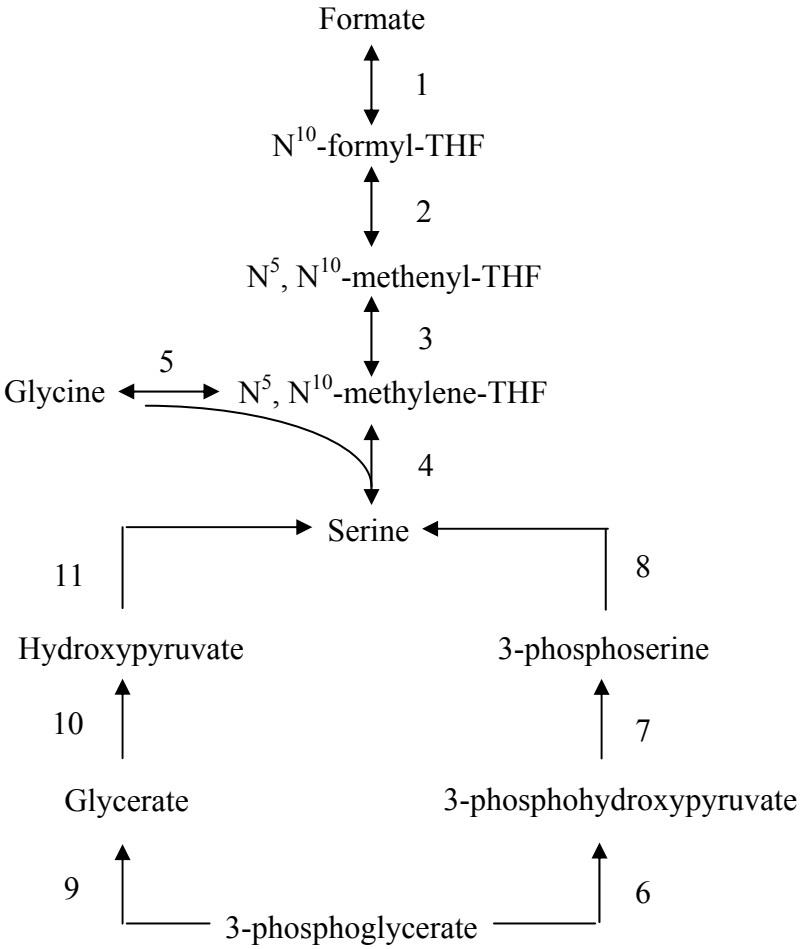

Fig. 2.2 Pathways of serine synthesis.

1. N^{10}-formyl-THF synthetase
2. N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase (THFC)
3. N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFD)
4. serine hydroxymethyltransferase (SHMT)
5. glycine decarboxylase complex (GDC)
6. 3-phosphoglycerate dehydrogenase (PGDH)
7. 3-phosphoserine aminotransferase (PSAT)
8. 3-phosphoserine phosphatase (PSP)
9. 3-phosphoglycerate phosphatase (PGP)
10. glycerate dehydrogenase (GDH)
11. alanine-hydroxypyruvate and glycine-hydroxypyruvate aminotransferase (AHAT and GHAT)

The phosphorylated pathway includes three enzymes: 3-phosphoglycerate dehydrogenase (PGDH), 3-phosphoserine aminotransferase (PSAT) and 3-phosphoserine phosphatase (PSP). PGDH catalyzes the oxidation of 3-phosphoglycerate to form 3-phosphohydroxypyruvate (reaction 6 and Fig. 2.2); PSAT is the second enzyme which catalyzes transamination from glutamate to α-ketoglutarate producing 3-phosphoserine from 3-phosphohydroxypyruvate (reaction 7 and Fig. 2.2); in the last step, 3-phosphoserine is dephosphorylated to serine by PSP (Ho and Saito, 2001; reaction 8 and Fig. 2.2).

$$\text{3-Phosphoglycerate} + NAD^{+} \xrightarrow{\text{PGDH}} \text{NADH} + \text{3-Phosphohydroxypyruvate} \quad (6)$$

$$\text{3-Phosphohydroxypyruvate} + \text{Glutamate} \xrightarrow{\text{PSAT}} \alpha\text{-ketoglutarate} + \text{3-Phosphoserine} \quad (7)$$

$$\text{3-Phosphoserine} + H_2O \xrightarrow{\text{PSP}} \text{Phosphate} + \text{Serine} \quad (8)$$

The non-phosphorylated pathway is initiated by dephosphorylation of 3-phosphoglycerate to form glycerate then hydroxypyruvate as intermediates. Four enzymes are involved: 3-phosphoglycerate phosphatase (PGP) (reaction 9 and Fig. 2.2), glycerate dehydrogenase (GDH) (reaction 10 and Fig. 2.2) and one or two aminotransferases, namely, alanine-hydroxypyruvate aminotransferase (AHAT) and glycine-hydroxypyruvate aminotransferase (GHAT) (Ho and Saito, 2001; reaction 11 and Fig. 2.2).

$$\text{3-Phosphoglycerate} + H_2O \xrightarrow{\text{PGP}} \text{Phosphate} + \text{Glycerate} \quad (9)$$

$$\text{Glycerate} + NAD^+ \xrightarrow{\text{GDH}} NADH + \text{Hydroxypyruvate} \quad (10)$$

$$\text{Hydroxypyruvate} + \text{Glycine} \xrightarrow{\text{AHAT, GHAT}} \text{Serine} + \text{Glyoxylate} \quad (11)$$

2.1.6 N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFC/THFD)

N^5, N^{10}-methylene-THF is a key branch point in THF-dependent C-1 metabolism. It can be utilized to synthesis serine, methionine and thymidylate or oxidized to form N^{10}-formyl-THF for purine synthesis by the sequential reactions of THFD and THFC.

A bifunctional NADP-dependent THFC/THFD has been found in *E. coli* (D'Ari and Rabinowitz, 1991) and *Clostridium thermoaceticum* (Ljungdahl et al., 1980). All of the prokaryotic enzymes exist as homodimers of subunit molecular mass 30-35 KDa (West et al., 1993). Developmental or differentiating mouse tissue cells and transformed human cells contain a mitochondrial bifunctional THFC/THFD with a subunit molecular mass of 34 KDa (Mejia and MacKenzie, 1985; Peri et al., 1989). West et al. (1993) cloned and characterized a *Saccharomyces cerevisiae* gene encoding THFD. This enzyme appears to be monofunctional which is different from other eukaryotic THFD. Disruption of the gene resulted in loss of enzyme activity and led to a purine requirement in certain genetic backgrounds, suggesting that this enzyme has a role in the oxidation of C-1 units for purine biosynthesis (West et al., 1993).

2.2 Cereal-powdery mildew pathosystem

Powdery mildew, one of the commonest plant diseases (Heitefuss, 2001), is most damaging if it appears three to four weeks before harvest and can result in yield losses exceeding 30% if left untreated. This disease also reduces photosynthetic ability, increases respiration and transpiration and impairs growth of host plants (Agrios, 1997). Cereals, such as wheat and barley, are severely affected by powdery mildew because of the difficulty of effectively controlling the disease by chemicals (Agrios, 1997). Cereal plants affected by this disease will decrease their number of grains per head and also lower their 1000-kernel weight (Agrios, 1997).

Mildew-infected plants typically have a greyish white, powdery coating over affected leaves and tissues, which then turn yellow-brown; finally, black cleistothecia may be present in the older areas of infection (Agrios, 1997).

Powdery mildew on wheat is caused by *Blumeria graminis* f. sp. t*ritici* (*Bgt*) which is an obligate biotroph. It is relatively easy to study the interaction between the host and pathogen because *Bgt* grows on the leaf surface. After 3-6 hours post inoculation (hpi), conidia of *Bgt* germinate on host leaves to produce a primary germ tube (PGT). Contact between the PGT and the leaf surface stimulates appressorial germ tube (AGT) formation, 6-12 hpi; then the AGT elongates to differentiate into a mature appressorium, 12-24 hpi. A penetration peg is produced from the appressorium, which then penetrates the host epidermal cell wall and produces a haustorium at 24-48 hpi.

The haustorium is formed inside the living plant cell and enables the fungus to obtain nutrients from its host. Sugars are transported into the haustorium by a sugar transporter located in the haustorial plasma membrane (Voegele et al., 2001) and amino acids are

transported by an amino acid transporter at the distal end of the haustorium (Hahn et al., 1997; Mendgen et al., 2000). The epidermal cells respond to the fungus by forming papillae, which can block further penetration of the infection. The epidermal cell responds to the PGT by forming a smaller papilla (3 μm in diameter) directly subtending PGT contact sites at 6 hpi and by forming a bigger papilla (5 μm in diameter) at sites of appressorium contact at 24 hpi (Thordal-Christensen et al., 2000). A papilla is a cell wall apposition located subjacent to the fungal germ tubes on the inner surface of the outer epidermal cell wall. It consists of several different components, including callose, phenylpropanoids, proteins, hydrogen peroxide and the specific chemical element, silicon (Thordal-Christensen et al., 1997; Carver et al., 1998; Thordal-Christensen et al., 2000). Inhibitor studies have demonstrated that callose and phenylpropanoids are directly involved in defense against infection (Thordal-Christensen et al., 2000). PGT can stimulate a series of localized host cell responses, including papilla deposition (Kunoh et al., 1977). Defense triggering by the PGT can explain how induced resistance can be obtained in the early stage of inoculation. Haustoria are never produced by the PGT but the success of the powdery mildews in infecting the host under dry conditions can be attributed to their ability to infect the host by their PGT. Because their PGT can take up water from the leaf surface, powdery mildew can colonize a host even during dry conditions, which is quite different from other fungi (Thordal-Christensen et al., 2000).

2.3 Defense-related genes in response to signal molecules

2.3.1 Polyamines in response to stresses

Polyamines not only play a key role in a series of developmental processes, such as rooth growth, floral initiation, somatic embryogenesis and the development of fruits (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990), but also involved in plant response to abiotic stresses, such as osmotic shock, potassium deficiency, drought and salt stress (Evans and Malmberg, 1989; Watson and Malmberg, 1996). Polyamine levels are also altered by pathogen infection (Geenland and lewis, 1984).

The first step in polyamine biosynthesis is the formation of putrescine which is synthesized from ornithine and arginine via ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). The polyamines spermidine and spermine are formed by the subsequent addition of an aminopropyl moiety to putrescine and spermidine respectively in reactions catalyzed by spermidine synthase and spermine synthase. The aminopropyl moiety is formed by the decarboxylation of AdoMet catalyzed by the enzyme SAMDC.

Polyamine metabolism will be changed in higher plants in response to a series of abiotic stresses. Krishnamurthy and Bhagwat (1984) indicated that salt-tolerant rice cultivars accumulated high levels of spermine and spermidine. In sorghum, spermine and spermidine accumulation occurred during salt-stress (Erdei et al., 1996). A gradual increase of ADC activity in the salt-tolerant rice cultivar was found by Chattopadhay et al. (1997). Lee et al., (1997) reported that activity of ADC and SAMDC and levels of spermine and spermidine in rice shoots increased after exposure to chilling. Osmotic treatments also induce high levels of putrescine and ADC in detached oat leaves (Flores and Galston, 1984).

Treatment of first leaves of barley with MeJA led to a significant reduction in powdery mildew infection in second leaves (Walters et al., 2002). The systemic protection was accompanied by significant increases in levels of putrescine and spermidine. Recently, MeJA has been shown to increase the expression of ADC gene in *Arabidopsis* (Perez-Amador et al., 2002).

Although relatively little work has been done on polyamines in response to microbial infection, Greenland and Lewis (1984) were the first to show that polyamine levels are changed after pathogen infection. They found that rust infection on barley resulted in an increased spermidine levels 6-7 times than that of healthy leaves. Walters et al. (2002) showed that levels of putrescine, spermine and spermidine were greatly increased in barley after inoculation with powdery mildew fungus. These changes were also accompanied by increased activity of ADC, ODC and SAMDC. Walters and Shuttleton (1985) indicated that infection of turnip roots by the clubroot fungus resulted in changes in concentrations of polyamines. Virus infection also leads to altered polyamine metabolism. Infection of tobacco by TMV leading to the hypersensitive reaction resulted in a 20-fold increase in ODC activity and increased concentrations of polyamines (Negrel eet al., 1984).

2.3.2 Enzymes in the lignin biosynthesis pathway

PAL is the first enzyme of the phenolic pathway and has been implicated in regulating lignification. The reaction catalyzed by PAL to produce trans-cinnamic acid is considered as a key step in the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Cinnamyl alcohol dehydrogenase (CAD) catalyzes the synthesis of cinnamyl

alcohols from their corresponding cinnamaldehydes and is considered to be a highly specific marker for lignification. OMT is involved in the biosynthesis of lignin precursors. The mRNAs encoding OMT are highly induced in tobacco leaves during the HR to TMV (Jaeck et al., 1992). Peroxidase has been proposed to participate in the latest steps of lignin biosynthesis and catalyzes the dehydrogenative polymerization of monolignols into lignins. Peroxidase associated with lignification has been described in several plants, such as tomato (Quiroga et al., 2000), poplar (Christensen et al., 2001), *Pinus* (Charvet-Candela et al., 2002) and wheat (Baga et al., 1995).

Lignin is one of the most abundant biopolymers, second only to cellulose in abundance on earth (Walter, 1992). It accounts for up to 30% of all vascular plant tissue (Croteau et al., 2000). It is deposited in secondary walls of lignifying tissues providing rigidity and structural support to the otherwise elastic polysaccharide cell walls (Walter, 1992). Zhong et al. (1997) showed that *Arabidopsis* mutants lacking lignified interfascicular fibres are no longer upright.

Lignified cell walls are considered to be very effective in limiting the progression of microorganisms and are resistant to degradation by most of them. This was demonstrated by the negative correlation between lignin content and digestibility of forages by ruminants (Walter, 1992). Moerschbacher et al. (1990) indicated that chemical inhibition of lignification in wheat resulted in a decreased resistance to *Puccinia graminis.*

Carver et al. (1991) showed that treatment of oat seedling leaves with the PAL inhibitors α-aminooxyacetate (AOA), α-aminooxy-β-phenylpropionic acid (AOPP) increased their quantitative susceptibility to *Erysiphe graminis* D.C. Two barley lines

Alg-S and RISØ-S, were treated with the PAL inhibitor, AOPP, and the CAD inhibitor, *N*-*O*-hydroxyphenyl-sulfinamoyl-tertiobutyl acetate (OH-PAS), resulting in an increased, quantitative susceptibility to powdery mildew (Carver et al., 1994).

Wounding can induce the synthesis and activity of PAL and other enzymes involved in phenylpropanoid metabolism, such as peroxidase and polyphenol oxidase (Ke and Saltveit, 1989). Wounding of potato leaves caused extremely rapid and transient induction of PAL mRNA (Joos and Hahlbrock, 1992). Campos-Vargas and Saltveit (2002) found that wounded lettuce produces a signal that induces the synthesis of PAL and the accumulation of phenolic compounds. The signal molecules JA, MeJA and SA can elevate PAL activity in younger leaves (Campos-Vargas and Saltveit, 2002). Creelman et al. (1992) reported that wounded soybean stems rapidly accumulated JA and MeJA. Chalcone synthase, vegetative storage protein and proline-rich cell wall protein mRNA levels were also increased after addition of MeJA to soybean suspension cultures (Creelman et al., 1992).

3. MATERIALS AND METHODS

3.1 Plant and fungal materials

The powdery mildew (*Blumeria graminis* f. sp. *tritici*) isolate was taken from the susceptible hexaploid wheat (*Triticum aestivum* L. cultivar Conway) in Saskatoon, Saskatchewan, Canada (Dr. Wei's lab) and was maintained on the same cultivar under greenhouse conditions. Over 150 accession lines of the diploid wheat (*Triticum monococcum* L.), with an AA genome (kindly provided by Dr. S. Fox, Cereal Research Center, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada) were screened for susceptibility to this isolate. Ten-day-old plants of one of the most susceptible and most resistant lines were used in this study and are referred to as Tm441 and Tm453, respectively. Plants were grown in enriched peat with dolomite in 10 × 10 cm square pots and grown in the same greenhouse room. The environmental conditions were controlled as follows: light period, 16 h, light intensity 150 $\mu Es^{-1}m^{-2}$ (Weston model 756 sunlight illumination meter, NewARK, N.J. USA), 24°C, approx. 60% relative humidity; dark period, 8 h, 18°C, approx. 70% relative humidity.

3.2 Biotic stress treatments

Ten seeds of Tm441 and Tm453 were sown in single rows in 36 pots for each accession line, then grown in the greenhouse with a cycle of 16 h light and 8 h

dark at 24°C day/18°C night for 10 days. The 10-day-old, fully expanded, primary leaves of Tm441 and Tm453 were then held down on a horizontal plexiglass plate to expose the abaxial epidermis for inoculation with *Bgt* (Wei et al., 1998; Fig. 3.1). The inoculation density was about 100 - 200 conidia per mm^2 achieved by shaking powdery mildewed Conway plants above the Tm441 and Tm453 leaves which were placed in an inoculation box. Tm441 and Tm453 plants were then incubated under the same conditions. To ensure that only the most vigorous and youngest conidia were used in experiments, leaves bearing colonies were shaken to remove old mildew conidia 48 h before inoculation.

Tm441 and Tm453 leaves infected with *Bgt* at 0, 1, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hpi were collected into 50 ml centrifuge tubes, immediately frozen in liquid nitrogen and then stored at -80°C for later total RNA extraction. Non-inoculated Tm441 and Tm453 leaves were used as control.

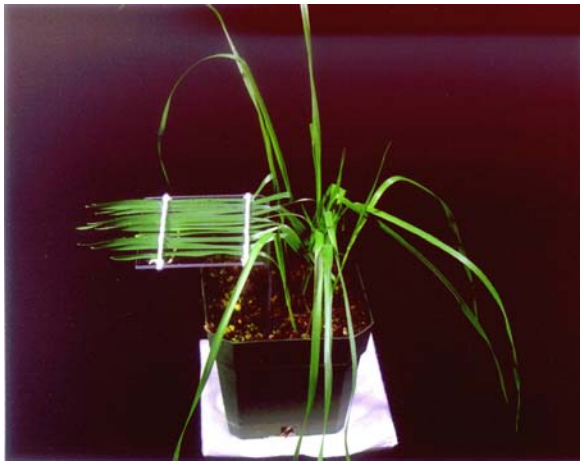

Fig. 3.1 Treatment of wheat plants with powdery mildew.

3.3 Abiotic stress treatments

3.3.1 Wounding treatment

Ten-day-old primary leaves of Tm441 plants were placed on a horizontal plexiglass plate and gently rubbed with powdered silicon carbide until slightly damaged. About 30 pieces of treated leaf were harvested at 1, 6, 12 and 24 h after treatment for total RNA extraction. Leaves from unwounded plants were used as controls.

3.3.2 Drought treatment

Tm441 plants, which had been grown in perlite, were harvested and the medium gently washed away. These plants were then transferred to trays inlaid with paper toweling. About 30 pieces of treated leaf were harvested at 1, 6, 12 and 24 h after the start of dehydration stress for total RNA extraction. Untreated plants were used as controls.

3.3.3 Cold treatment

Cold stress was performed by transferring Tm441 plants to a dark chamber at 4°C. Total RNA from 30 pieces of treated leaf were harvested at 1, 6, 12, 24 and 48 h after treatment. Control plants grown at 25°C in a dark chamber were sampled at the same times.

3.3.4 Sodium chloride treatment

Ten-day-old plants of Tm441, which had been grown in perlite, were harvested and the medium gently washed away. The plants were floated in 250 mM NaCl solution. Approximately 30 pieces of treated leaf were harvested at 1, 12, 24, 48 h after treatment

for total RNA extraction. Plants floated in water were used as control and harvested at the same time as the stressed plants.

3.4 Treatments with stress signaling molecules

Ten-day-old Tm441 plants were sprayed with solutions of 0.125% triton X-100 containing one of the following elicitors: 10 mM H_2O_2 (Curtis et al., 1997), 100 μM ethephon (releasing ethylene; Hiraga et al., 2000), 200 μM methyl jasmonate (MeJA; Agrawal et al., 2002), 5 mM salicylic acid (SA; Curtis et al., 1997), 100 μM abscisic acid (ABA; Curtis et al., 1997), 10 mM sodium nitroferricyanide (Ш) dihydrate (releasing nitric oxide (NO); Delledonne et al., 2001). Total RNA from 30 pieces of treated leaf were collected at 1, 6, 12 and 24 h after spraying. Plants sprayed with distilled water with 0.125% triton X-100 were used as controls.

3.5 cDNA library construction and expressed sequence tag (EST) analysis

3.5.1 cDNA library construction

A cDNA library was previously constructed from the total Poly(A)$^+$RNA isolated from the diploid wheat line 441 leaf epidermis infected with *Bgt* 24 h after inoculation, and named the 441E-cDNA library (Wei, unpublished). Total RNA was extracted according to Wilkins and Smart (1996); Poly(A)$^+$RNA was isolated using the Promega PolyA Ttract® mRNA system. The λZAPП cDNA library protocol (Stratagene) was used for the construction of the cDNA library.

3.5.2 Plasmid extraction

Plasmid DNA used to sequence and prepare probes used in northern hybridization was extracted by a standard protocol (Sambrook and Russell, 2001). Bacterial culture (3 ml) in LB medium (1% NaCl, 1% Tryptone, 0.5% Yeast extract) supplemented with the antibiotic, ampicillin (50 µg/ml), was grown overnight. An aliquot (1.5 ml) of culture was transferred to a microcentrifuge tube and spun at 14,000 rpm for 1 min in an Eppendorf centrifuge. The bacterial pellet was resuspended in 300 µl of Resuspension Buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNAase A) and lysed with 300 µl of Lysis Buffer (200 mM NaOH, 1% SDS). The tube was inverted 4-6 times to mix the solution gently and thoroughly. After incubation at room temperature for 5 min, 300 µl chilled Neutralization Buffer (3.0 M potassium acetate, pH 5.5) were added to the tube. After brief mixing by inversion, the mixture was centrifuged at 14,000 rpm for 10 min. The supernatant containing the DNA (approximately 750 µl) was precipitated with the same volume of isopropanol at room temperature. The mixture was inverted several times, precipitated at room temperature for 15 min and then centrifuged at 14,000 rpm for 10 min. The supernatant was discarded, the pellet washed once with 500 µl 70% ethanol and then centrifuged at 14,000 rpm for a further 10 min. The DNA pellet was dried at room temperature for 20 min and resuspended in 50 µl sterile ddH_2O, then stored at -20°C. Restriction enzyme digestion and DNA sequencing were used to identify the plasmid DNA. The DNA size was determined by a 100-bp DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA).

3.5.3 DNA digestion

Restriction enzymes and their buffers were purchased from Invitrogen Life Technologies. A typical reaction consisted of 4 µl of the One-Phor-All 10 × Buffer, 2.5 µl *Eco*RI, 2.5 µl *Xho*I, 16 µl ddH_2O and 15 µl of extracted plasmid DNA. The DNA was then digested at 37°C for 2 h, after which about 40 µl of it and 10 µl 6 × DNA Loading Buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water) were loaded on a 1.2% agarose gel. The DNA fragments were separated at 70 v for about 1 h in TAE Buffer (2 M Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH8.0).

3.5.4 DNA purification

Each DNA fragment was excised from the agarose gel with a clean scalpel, chopped into fine pieces and purified using the Qiaquick kit (Qiagen). Three volumes of Buffer QG (Qiagen) were added to 1 volume of agarose gel and incubated at 50°C for 10 min until the gel slice was completely dissolved. The entire volume was applied to the Qiaquick column and centrifuged at 14,000 rpm for 1 min. The flow-through solution was discarded and the column placed back in the same collection tube. The column containing the DNA was washed with 750 µl Buffer PE (Qiagen), left standing for 2 min and then centrifuged at 14,000 rpm for 1 min. The flow-through solution was discarded and an additional centrifugation at 14,000 rpm for 1min was performed to ensure that the residual ethanol from Buffer PE was completely removed. The DNA was eluted by adding 50 µl Elution Buffer (Qiagen) and centrifuged at 14,000 rpm for 1min. The purified DNA was stored at -20°C for use in the labeling of probes. The concentration of

the DNA was determined by comparison with a 30 ng 4 kilobase (kB) standard prepared from digested pBI 221 plasmid DNA.

3.5.5 DNA sequencing and EST analysis

5'- end cDNA sequencing (using an ABI 377 automated sequencer at the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan) for 2700 clones was performed previously (Wei and Selvaraj, unpublished). To get the full-length cDNA, sequencing was performed by using T3 forward primers through an ABI 377 sequencer. Primers were designed and synthesized based on the known 5' sequence (Table 3.1). As all of the cDNAs contained 3' polyA tail, cDNAs which had 5' ATG that aligned with the start codons of genes known to encode homologues of the gene of interest were assumed to be full-length.

Nucleotide sequences were analyzed by comparison with the databases using Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/blastx). The deduced amino acid sequence of the cDNA was analyzed by the program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted protein localization was obtained from (http://psort.nibb.ac.jp/form.html) and (http://www.cbs.dtu.dk/services/TargetP). Multiple sequence alignment was performed by the program (http://searchlauncher.bcm.tmc.edu). A phylogenetic tree was constructed based on ClustalW 1.81 alignment (http://clustalw.genome.jp).

Table 3.1 List of primers used for full-length EST sequences

Amplified gene	Primer	Oligo sequences (5'- 3')
Tm-MTHFR	2669 F1	ATAACTGTCGCTGGCTATCCA
	2669 F2	ATACGGTGCACTTAATGATC
	2669 F3	ATGAAGCGTTTGAGATCTGG
	2669 F4	AATTTAGCAACCTAAATTAG
Tm-AdoMet Syn2661	2661 F1	AGTCTCCTGACATTGCCCA
Tm-AdoMet Syn605	605 F1	AAGATCATCATCGACACCTAT
Tm-SAMDC	886 F1	GCTTGCTGCTTCACGATAAT
	886 F2	TCTTCTTCAAGACTCAAGCT
	886 F3	AAGCTTGCCAACCTCCTCGCAT
	886 F4	GATTGATTCATCATACAATA
Tm-SHMT	1139 F1	ACATCTTTCCCATGGTTACC
	1139 F2	AATAGATGGTTCACGAGTG

3.6 Manipulation of wheat ribonucleic acids

3.6.1 RNA isolation

Total RNA was extracted using the phenol/chloroform protocol revised according to Wilkins and Smart (1996).

Approximately 3 g of plant material were ground thoroughly in liquid nitrogen in a mortar and pestle and then transferring to a 50 ml Falcon centrifuge tube containing 15 ml Extracting Buffer (150 mM LiCl ; 50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 5% SDS). After vortexing, a phenol/chloroform mixture (500 g phenol melted in 150 ml water, 0.5 g 8-hydroxyquinoline, 500 ml chloroform) (15 ml) was added and mixed well. The tube was centrifuged at 2000 rpm for 15 min at 4°C and the supernatant transferred to a new tube containing 15 ml of phenol/chloroform. The tube was centrifuged again at 2000 rpm for 15 min at 4°C, a step repeated 4 times. The supernatant was then transferred to a new tube with 15 ml chloroform added,

centrifuged a final time at 2000 rpm for 15 min at 4°C and the supernatant transferred to a 30 ml Corex tube (sterilized at 180°C for 4 h). The RNA/DNA mix was precipitated by adding 1/12 vol 4 M NaAc, pH 5.5, and 0.8 vol isopropanol overnight at -20°C. The tube was centrifuged at 8000 rpm for 30 min at 4°C and the pellet dissolved in 5 ml ice-cold ddH_2O. RNA was precipitated by adding 2 M LiCl and incubating on ice for 1 h. The tube was again centrifuged at 8000 rpm for 30 min at 4°C; this time the pellet was transferred to a sterile 1.5 ml microcentrifuge tube using 70% ice-cold ethanol and then centrifuged at 14000 rpm for 15 min at 4°C. The pellet was dried on ice and then dissolved in 100 µl sterile ddH_2O and kept at -80°C. The purity and concentration of the RNA were estimated by spectrophotometer readings at OD_{260} and OD_{280}. All the samples had an OD_{260}/OD_{280} ratio in the range 1.7 - 2.0, which showed that the RNA was only minimally contaminated with protein.

3.6.2 RNA blot

Twenty micrograms of total RNA in a final volume of 5 µl of sterile ddH_2O was mixed with 15 µl of RNA Loading Buffer (465 µl deionised formamid, 100 µl 10 × MOPS, 135 µl 37% formaldehyde, 50 µl 88% glycerol, 1 µg bromophenol). The sample was boiled for 3 min, cooled on ice, then loaded on a 1.2% formaldehyde agarose gel (1.2% agarose, 1% formaldehyde, 1 × MOPS) and electrophoresed in 1 × MOPS Buffer (10 × MOPS Buffer containing 200 mM MOPS, 50 mM NaAc, 10 mM EDTA, pH 7.0) at 65 v for 4 h. Before transfer of the RNA, a photograph of the gel was taken to make sure that the RNA was not degraded. Before blotting, the gel was shaken twice in an excess of ddH_2O at room temperature for 15 min each time. The Zetaprobe membrane

(BioRad, CA) was wetted in ddH_2O for 15 min and then soaked in 20 × SSC (3 M NaCl, 0.3 M Na Citrate) for another 5 min. The RNA was transferred from the gel to the Zetaprobe membrane overnight in 20 × SSC transfer buffer: The transfer buffer was drawn from a reservoir through the gel and into a stack of paper towel. The nucleic acid was eluted from the gel in the moving stream of buffer and deposited on the Zetaprobe membrane. A glass plate and a weight added on top of the paper towel ensured tight connections in the transfer system sandwich. After blotting, the membrane was washed twice in 2 × SSC for 10 min each and stained with 0.02% methylene blue solution (0.02% methylene blue in 0.3 M NaAc, pH 5.5) to confirm the even loading of ribosomal RNA onto the membrane. The wet membrane was photographed by using a ruler to show locations of the RNA, baked at 80°C for 2 h to cross-link to the RNA (Sambrook and Russell, 2001) and finally stored in a plastic bag at 4°C until used for hybridization.

3.6.3 Prehybridization and hybridization

The Zetaprobe membrane containing the RNA was prehybridized at 65°C in a hybridization oven (Model 5400, VWR) for 3 h in Hybridization Buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, 7% SDS, pH 7.4).

For probe preparation, plasmid DNA was digested with *Eco*RI and *Xho*I at 37°C for 2 h, run in a 1.2% agarose gel and then extracted using a Qiaquick Gel Extraction Kit. About 100 ng of cloned cDNA were labeled with ^{32}P using the Amersham Megaprime DNA labeling system. The labeling reaction mixture, which included 100 ng of template, a 5µl mixture of dGTP, dATP, dTTP primers and the appropriate volume of water to give a total volume of 50 µl in the final Megaprime reaction, was denatured at 95 -

100°C for 5 minutes in a boiling water bath and immediately cooled on ice. Ten microliters of labeling buffer, 2 µl of Klenow enzyme (1U/µl) and 5 µl of α-^{32}P dCTP was added and mixed well. The mixture was incubated at 37°C for 2 h and the reaction then stopped by adding 5 µl of 0.2 M EDTA. The radioactivity of the labeled probe was determined with a Contamination Meter (Model TBM-3S, Canoga Park, CA, USA).

The labeled probe was purified from unincorporated α-^{32}P dCTP by using a Pharmacia NICK column. The top cap of the column was removed, the liquid poured off and the column then rinsed once with Equilibration Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The bottom cap was removed and the column supported over a suitable receptacle and approximately 3 ml of the equilibration was added to equilibrate the gel. After the Equilibration Buffer had completely entered the gel bed, the above labeling reaction solution was added followed by 400 µl Equilibration Buffer. After the liquid had completely entered the gel bed, another 400 µl of Equilibration Buffer was added to purify the labeling DNA. The purified sample with 400 µl of Equilibration Buffer was collected for later use. The radioactivity was measured, and found to be about 100 × 400 counts per minute (CPM). Probes with a labeling efficiency over 50% were used for further hybridization.

The labeled DNA was denatured by heating to 95 - 100°C for 5 minutes and then immediately cooled on ice. Labeled probe was added to the hybridization tube and hybridization carried out at 65°C overnight in Hybridization Buffer. After hybridization, the membrane was washed twice for a total of 40 min at 65°C in Wash Buffer 1 (2 × SSC solution containing 0.1% SDS) , followed by a further two washes for a total of 40 min at 65°C in Wash Buffer 2 (0.1 × SSC solution containing 0.1% SDS). After washing,

the membrane was put into a plastic wrap and exposed to Kodak X-ray film at -80°C in an X-ray film cassette to minimize non-specific exposure. The film was developed and fixed by Kodak GBX developer and fixer several days to two weeks later, depending on the signal intensity.

For RNA quantification, blots were scanned and analyzed by spot densitometry using Alphalmager^{TM} 2200 Documentation and Analysis System (Alpha Innotech, San Leandro, CA, USA). The results are expressed as average value of pixels enclosed after background correction divided by area.

4. RESULTS

4.1 Gene identification in wheat Tm441 cDNA library by EST analysis

A 441E-cDNA library was constructed from the total Poly(A)$^+$RNA isolated from the diploid wheat line 441 (*Triticum monococcum* with an AA genome, the susceptible line) leaf epidermis highly infected with *B. graminis* f. sp. *tritici* 24 hours after inoculation (Wei, unpublished). The library contains cDNA populations derived from both the plant tissue and the pathogen. About 2700 clones were sequenced from the 5' terminus to produce an EST collection (Wei and Selvaraj, unpublished results). The BLASTX program was used to query the National Center for Biotechnology Information (NCBI) database to identify genes of interest. The BLASTX can translate the cDNA sequence into protein sequences in six open reading frames (ORFs) and compare the sequences to the database. After blasting, the genes linked to the generation and supply of methyl units were selected and used for analyses in this project. Of 2700 ESTs analyzed, one clone showed high similarity to MTHFR, two clones to Met Syn, two clones to AdoMet Syn, seven clones to SAMDC, three clones to SHMT, two clones to THFC/THFD, two clones to AdoHcy Hy, one clone to ACC oxidase, one clone to NA synthase, one clone to CCoAOMT, one clone to COMT and one clone to Arginine methyltransferase were indentified (Table 4.1).

Table 4.1 List of T. monococcum cDNA clones in the pathways of generation and supply of methyl units

Genes	Number	cDNA clones
N^5, N^{10}-methylenetetrahydrofolate reductase	1	441E-2669
cobalamin-independent methionine synthase	2	441E-397 441E-2410 (fusion gene)
S-adenosylmethionine synthetase	2	441E-2661 (isoform) 441E-605 (isoform)
S-adenosylmethionine decarboxylase	7	441E-693 (identical) 441E-1110 (identical) 441E-886 (identical) 441E-2594 (identical) 441E-2621 (identical) 441E-844 (fusion gene) 441E-1233 (fusion gene)
serine hydroxymethyltransferase	3	441E-660 (identical) 441E-1139 (identical) 441E-2776 (identical)
N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase	2	441E-458 441E-2341 (fusion gene)
S-adenosylhomocysteine hydrolase	2	441E-483 441E-491 (fusion gene)
1-aminocyclopropane-1-carboxylate oxidase	1	441E-2779
Nicotianamine synthase	1	441E-2535
Caffeoyl CoA *O*-methyltransferase	1	441E-924
Caffeic acid *O*-methyltransferase	1	441E-2269
Arginine methyltransferase	1	441E-635

4.2 Isolation and characterization of genes linked to the generation and supply of methyl units

4.2.1 N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR)

In the present study, a full-length cDNA clone 441E-2669 encoding a putative MTHFR was identified from the 441E-cDNA library and designated *Tm-MTHFR*. The 2374 bp nucleotide sequence of *Tm-MTHFR* contained a 88 bp 5' noncoding region, a 540 bp 3' untranslated region, an ORF of 1746 nucleotides which encoded a putative protein of 582 amino acids (Fig. 4.1). The predicted Tm-MTHFR protein has an estimated molecular mass of 64.9 KDa and an isoelectric point (pI) of 5.86.

The amino acid sequence of the putative Tm-MTHFR is based on conceptual translation of these DNA sequences. Searches in GenBank performed with the full-length deduced Tm-MTHFR amino acid sequence revealed that Tm-MTHFR had high similarity to several plant genes, including an 86.6% sequence identity to *Zea mays* (Zm, GenBank accession number AAD51733, Roje et al., 1999), *Oryza sativa* (88.0%) (Os, XP_470089), *Arabidopsis thaliana1* (76.5%) (At1, AAC23420), *Arabidopsis thaliana2* (76.3%) (At2, CAB53783), also 39.2% identity to *Homo sapiens* (Hs, CAB41971), *Saccharomyces cerevisiae* (36.9%) (Sc, P53128, Tizon et al., 1996) and *Escherichia coli* (28.7%) (Ec, P00394, Saint-Girons et al., 1983) (Fig. 4.2). There are 19 residues that interact with the FAD cofactor in the N-terminal catalytic domain of the *E. coli* enzyme (Fig. 4.2, asterisks), among which, 17 are identical or conservatively replaced in the plant sequences. The highly-charged sequence KRREED is predicted to have the highest hydrophilicity in the deduced human sequence (Fig. 4.2, blue bar). In human, the

C-terminal domain contains a putative AdoMet binding site SYIYRTQEWDEFPNGRWGNSS (Fig. 4.2, red bar).

Phylogenetic analysis was used to compare the amino acid sequences of Tm-MTHFR with other MTHFRs (Fig. 4.3). The results showed that Tm-MTHFR has high degree of identity to monocots *O. sativa* (88.0%) and *Z. mays* (86.6%) and low degree of identity to *H. sapiens* (39.2%), *S. cerevisiae* (36.9%) and *E. coli* (28.7%).

The subcellular localization predicted by using ChloroP, MITOPROT, TargetP (Emanuelsson et al., 2000) and PSORT showed that the predicted amino acid sequence of Tm-MTHFR contained no transit peptide for chloroplastic or mitochondrial localization, suggesting that *MTHFR* may encode a cytosolic MTHFR.

```
ccattagcagagcagtgggggagaaggttctagaggaagaggaagaggaagatgaaggtga  -28
tcgagaagatccaggaggcggcggcga atg gccggaccgtcttctccttcgagtacttcc   33
                            M  A  G  P  S  S  P  S  S  T  S      11
cgcccaagacggagggagggcgtggagaacctcttcgagcggatggaccgcatggtggcg    93
R  P  R  R  R  E  G  V  E  N  L  F  E  R  M  D  R  M  V  A      31
cacggccccaacttctgcgacatcacctggggcgccggcggatccaccgccgacgtcacc   153
H  G  P  N  F  C  D  I  T  W  G  A  G  G  S  T  A  D  V  T      51
ctcgacatcgccaaccgcatgcagaacatggtatgcgtggaaacgatgatgcacttgaca   213
L  D  I  A  N  R  M  Q  N  M  V  C  V  E  T  M  M  H  L  T      71
tgcaccaacatgccagtggagaagatcgataatgctttggataccatcaagtccaatggg   273
C  T  N  M  P  V  E  K  I  D  N  A  L  D  T  I  K  S  N  G      91
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I  Q  N  V  L  A  L  R  G  D  P  P  H  G  Q  D  K  F  V  Q     111
gttgctggtggattttcttgtgctctagatctggtggagcacattaaagccaagtatggt   393
V  A  G  G  F  S  C  A  L  D  L  V  E  H  I  K  A  K  Y  G     131
gattactttggcataactgtcgctggctatccagaggcacaccctgaggtaatactaggc   453
D  Y  F  G  I  T  V  A  G  Y  P  E  A  H  P  E  V  I  L  G     151
gaggaaggtgctacggaggaagcatataggaaagatcttgcttacttgaagagaaaggtt   513
E  E  G  A  T  E  E  A  Y  R  K  D  L  A  Y  L  K  R  K  V     171
gatgctggcgctgacgttatagtcacccagcttttctatgataccgatatctttctcaag   573
D  A  G  A  D  V  I  V  T  Q  L  F  Y  D  T  D  I  F  L  K     191
tttgtgaacgactgccgtcagattggtataacctgccctatcgttcctggcataatgcca   633
F  V  N  D  C  R  Q  I  G  I  T  C  P  I  V  P  G  I  M  P     211
ataaataactacaaaggatttgtgcgcatgactggattctgcaaaactaagattccagct   693
I  N  N  Y  K  G  F  V  R  M  T  G  F  C  K  T  K  I  P  A     231
gagattactgctgccttggatcctattaaagacaatgaggaggctgtgaaagcatatgga   753
E  I  T  A  A  L  D  P  I  K  D  N  E  E  A  V  K  A  Y  G     251
atccaccttggtactgagatgtgcaagaaaattttggctagtgggatcaagactttgcac   813
I  H  L  G  T  E  M  C  K  K  I  L  A  S  G  I  K  T  L  H     271
ctgtacacactaaacatggagaagactgctttagcaattctgatgaatcttggattaata   873
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L  Y  T  L  N  M  E  K  T  A  L  A  I  L  M  N  L  G  L  I     291
gaggagtccaagctttcaagaacattaccttggaggccaccaactaatgttttccgtgtc    933
E  E  S  K  L  S  R  T  L  P  W  R  P  P  T  N  V  F  R  V     311
aaagaggatgttcgccctatattctgggccaacagaccaaagagttacatttcaaggacc    993
K  E  D  V  R  P  I  F  W  A  N  R  P  K  S  Y  I  S  R  T     331
actggttgggatcaatacccacatggacggtggggtgattccaggaacccatcatacggt   1053
T  G  W  D  Q  Y  P  H  G  R  W  G  D  S  R  N  P  S  Y  G     351
gcacttaatgatcaccagttcacacggccacgtggacgtggtaagaagctccaagaggaa   1113
A  L  N  D  H  Q  F  T  R  P  R  G  R  G  K  K  L  Q  E  E     371
tgggctgttccactgaaatctgtgcaagacattaatgagcggttcgtgaacttctgtgaa   1173
W  A  V  P  L  K  S  V  Q  D  I  N  E  R  F  V  N  F  C  E     391
ggaaaacttaaaagcagcccatggtctgagttagatggtcttcaacctgagacgacgata   1233
G  K  L  K  S  S  P  W  S  E  L  D  G  L  Q  P  E  T  T  I     411
attgacgatcagctggtgaagattaactcaaagggtttccttaccatcaacagccaacct   1293
I  D  D  Q  L  V  K  I  N  S  K  G  F  L  T  I  N  S  Q  P     431
gctgtaaatgcagagaaatctgagtctcctagtgttggatggggcggcccaggaggctat   1353
A  V  N  A  E  K  S  E  S  P  S  V  G  W  G  G  P  G  G  Y     451
gtttaccagaaggcctacgtcgaattcttctgcgctaaggagaagctgggccaactcatc   1413
V  Y  Q  K  A  Y  V  E  F  F  C  A  K  E  K  L  G  Q  L  I     471
gagaagagcaaggcattcccttccctcacgtacatcgccgtgaacaaggaaggagaatcg   1473
E  K  S  K  A  F  P  S  L  T  Y  I  A  V  N  K  E  G  E  S     491
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I  S  N  I  P  A  N  A  V  N  A  V  T  W  G  V  F  P  G  K     511
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E  I  I  Q  P  T  V  V  D  S  A  S  F  M  V  W  K  D  E  A     531
tttgagatctggtccaggggatgggcctgcctgttcccagagggcgactcgtccagggag   1653
F  E  I  W  S  R  G  W  A  C  L  F  P  E  G  D  S  S  R  E     551
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L  L  E  Q  I  Q  K  S  Y  Y  L  V  S  L  V  D  N  D  Y  I     571
agcggggacctctttgctgcattcaaggagatctaatttcgatgagaccttacagtatgc   1773
S  G  D  L  F  A  A  F  K  E  I  stop                          582
tgcgtttgaccgcccttcgctagagtcctgtaatatgatttgtcgtgatttctgtcgatt   1833
tatccaaaccactctatgaataagaatttctatctgtgttccttcgtgtgcagtctctc    1893
cacttgtctagcctatttgttacctaggtttattttacccaatgatgagttgaagctttt   1953
ttaatgtatatacgtggtaatacagtttactgccacgacagaagctcggttcattacttg   2013
ggttataatttcaaatgagaaccactcatctatgaaggattgaagatgtcaactagcgag   2073
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attataagttctctgcaaatgtaactaactgaagatcatatatgataagcaatttagcaa   2193
cctaaattagttataaaagtaataagagataggaaaaagtaaagttggcatgagtgatga   2253
tgatctctaagttcatttccttggagggaagctaaaaaaaaaaaaaaaaaaaaaaa      2310
```
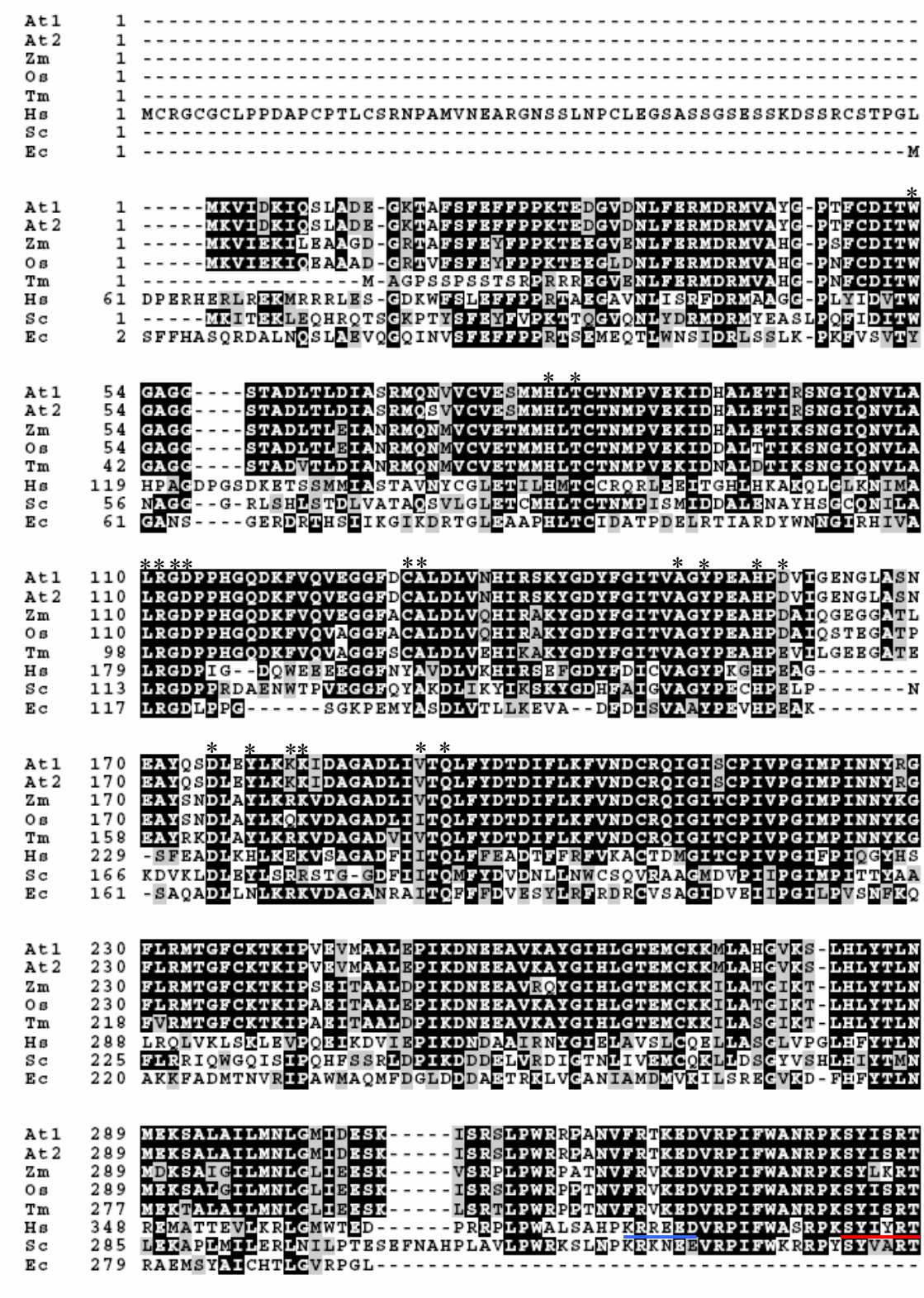

Fig. 4.1 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-MTHFR. Numbers in the right margin refer to the base pair and amino acid positions. Start and stop codons are in *bold.*



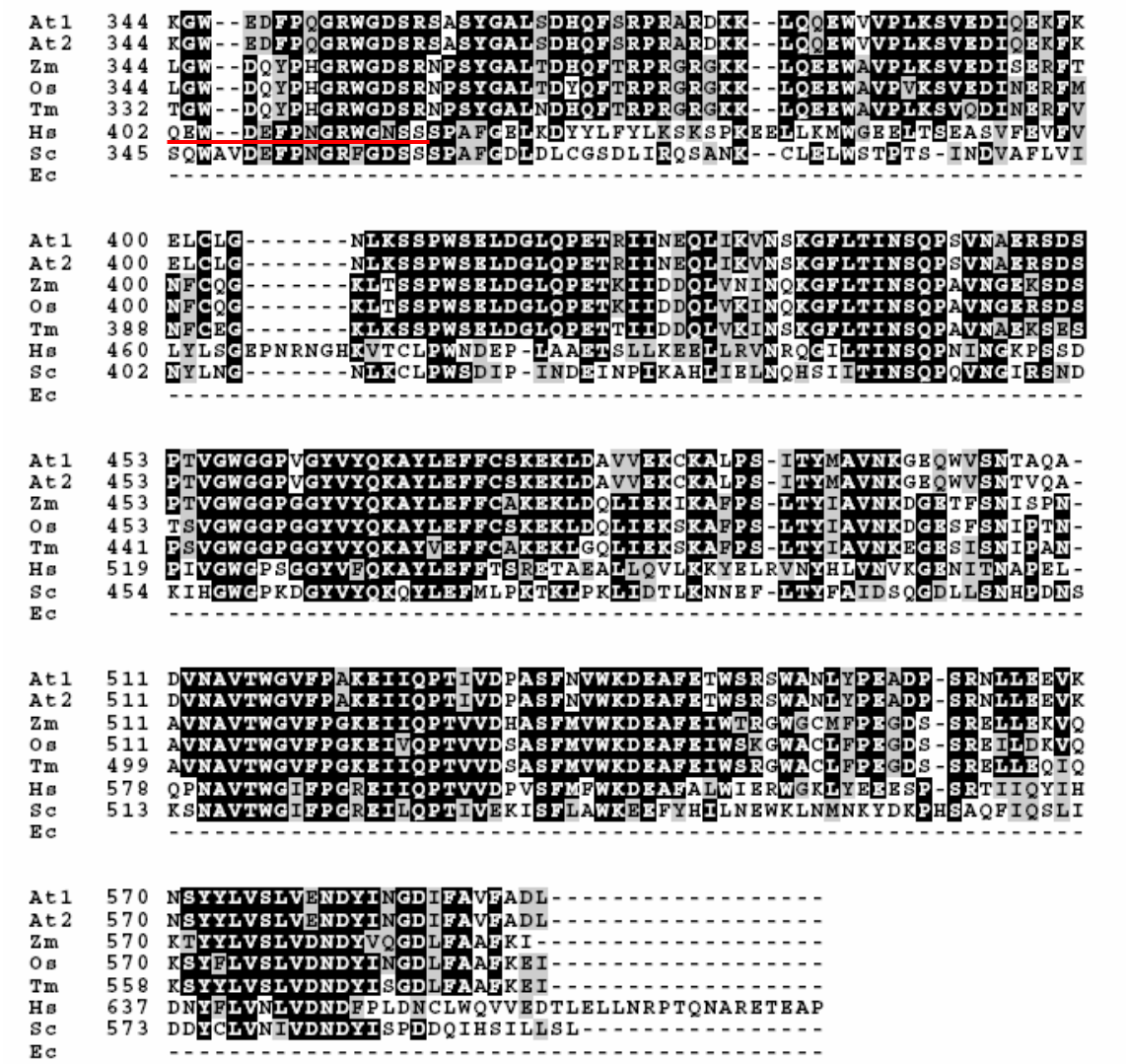


Fig. 4.2 Alignment of the deduced amino acid sequences of plant MTHFRs with those from *Homo sapiens, Saccharomyces cerevisiae* and *Escherichia coli*. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Zm, *Zea mays* (AAD51733, Roje et al., 1999); Os, *Oryza sativa* (XP_470089); At1 and 2, *Arabidopsis thaliana* (AAC23420, CAB53783, respectively); Hs, *Homo sapiens* (CAB41971); Sc, *Saccharomyces cerevisiae* (P53128, Tizon et al., 1996); Ec, *Escherichia coli* (P00394, Saint-Girons et al., 1983). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. *Asterisks* indicate residues that interact with the FAD prosthetic group in Ec (Guenther et al., 1999). The *blue bar* shows the hydrophilic bridge region between the domains in *Homo sapiens* (Goyette et al., 1994). The *red bar* indicates the putative AdoMet binding sequence in *Homo sapiens* (Goyette et al., 1994).

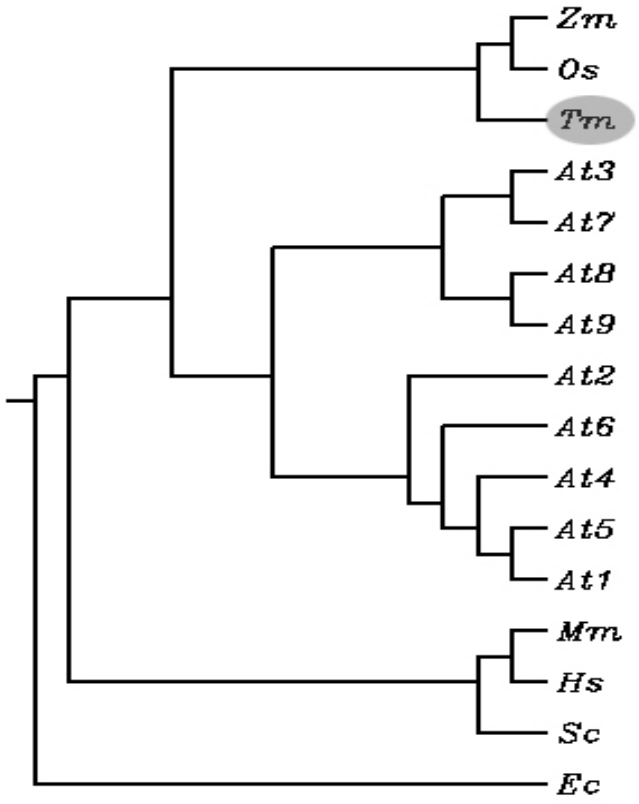


Fig. 4.3 Phylogenetic tree of MTHFRs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Zm, *Zea mays* (AAD51733, Roje et al., 1999); Os, *Oryza sativa* (XP_470089); At1-9, *Arabidopsis thaliana* (AAC23420, CAB53783, AAD55787 (Roje et al., 1999), AAD55788 (Roje et al., 1999), AAK43892, T00696, AAM67455, NP_850724, NP_850723, respectively); Mm, *Mus musculus* (AAD20313, Goyette et al., 1998); Hs, *Homo sapiens* (CAB41971); Sc, *Saccharomyces cerevisiae* (P53128, Tizon et al., 1996); EC, *Escherichia coli* (P00394, Saint-Girons et al., 1983).

4.2.2 Cobalamin-independent methionine synthase (Met Syn)

A partial cDNA clone 441E-397 encoding a putative Met Syn was isolated from the 441E-cDNA library and named *Tm-Met Syn*. The *Solanum tuberosum* Met Syn has been estimated to be 2644 bp (Zeh et al., 2002). The N-terminal amino acid of the deduced polypeptide from wheat aligns with position 538 of the *Solanum tuberosum* protein. Therefore, if the two have similar size, sequence coding for >530 amino acids are missing on the Tm-Met Syn cDNA. A C-terminal motif WVNPDCGLKTR is present and the cysteine residue in this motif functions as a zinc ligand (González et al., 1996) (Fig. 4.4).

Comparison of the Tm-Met Syn deduced amino acid sequence with those from other sources demonstrated that the Tm-Met Syn sequence had high similarity to several, including *Hordeum vulgare* (100%) (Hv, GenBank accession number BAD34660); *Sorghum bicolor* (93.4%) (Sb, AAL73979), *Zea mays* (90.8%) (Zm, AAL33589), *Solanum tuberosum* (89.0%) (St, AAF74983); *Catharanthus roseus* (89.0%) (Cr, CAA58474, Eichel et al., 1995), *Arabidopsis thaliana1* (83.8%) (At1-chl, CAE55865, Ravanel et al., 2004) and *Escherichia coli* (56.1%) (Ec, AAA23544, González et al., 1992) (Fig. 4.5). A C-terminal motif seems to be strictly conserved in Met Syn from plants and *E. coli.* (Fig. 4.5, black bar). At1-chl contains an N-terminal signal peptide sequence, which targets to chloroplastic localization (Fig. 4.5).

Furthermore, the phylogenetic tree analysis showed that Tm-Met Syn is grouped into cytosolic Met Syns, which is quite distant from the chloroplastic Met Syns cluster (Fig. 4.6).

```
accgtcttctggtccaagatggcacagagcatgactgctcgcccaatgaagggaatgttg    60
T  V  F  W  S  K  M  A  Q  S  M  T  A  R  P  M  K  G  M  L     20
acaggccctgtcacaatccttaactggtcttttgtcagaaatgaccaaccgaggtttgag   120
T  G  P  V  T  I  L  N  W  S  F  V  R  N  D  Q  P  R  F  E     40
acttgctaccagattgctcttgcaatcaagaaggaggtcgaggatcttgaggctggtggt   180
T  C  Y  Q  I  A  L  A  I  K  K  E  V  E  D  L  E  A  G  G     60
attcaggtcatccaaattgacgaggctgctttgagagagggtctgccactccgcaagtcc   240
I  Q  V  I  Q  I  D  E  A  A  L  R  E  G  L  P  L  R  K  S     80
gagcacgctttctacttggactgggccgtgcactccttcaggatcaccaactgcggtgtc   300
E  H  A  F  Y  L  D  W  A  V  H  S  F  R  I  T  N  C  G  V    100
caggacaccacccagatccacacccacatgtgctactccaacttcaacgacatcatccag   360
Q  D  T  T  Q  I  H  T  H  M  C  Y  S  N  F  N  D  I  I  Q    120
tccatcatcaacatggatgctgatgtgatcaccatcgagaactcacggtccgacgagaag   420
S  I  I  N  M  D  A  D  V  I  T  I  E  N  S  R  S  D  E  K    140
cttctctccgtcttccgcgagggtgtggtgtacggtgctggcattggcccgggtgtgtac   480
L  L  S  V  F  R  E  G  V  V  Y  G  A  G  I  G  P  G  V  Y    160
gacatccactcccccaggatcccttccaaggaggagatcgccgaccgtgtcaacaagatg   540
D  I  H  S  P  R  I  P  S  K  E  E  I  A  D  R  V  N  K  M    180
ctcgcggtcctcgacaccaacatcctgtgggtgaaccccgactgcggtctcaagacccgc   600
L  A  V  L  D  T  N  I  L  W  V  N  P  D  C  G  L  K  T  R    200
aagtacgccgaggtcaagcctgccctcaccaacatggttgaggctgccaagcagatccgt   660
K  Y  A  E  V  K  P  A  L  T  N  M  V  E  A  A  K  Q  I  R    220
gccgagctcgccaaggcgcagtaagccgtgctcgtatagcagctccccccatttaattg   720
A  E  L  A  K  A  Q  stop                                      227
caaggaggatgtcaccaccatagccgtgtttactttgaataatctgggtctcgtatccac   780
ttgttggctaggctagtttgtttctcggcacgatgtgctcatcccttgatttggtggttt   840
ttgaggcggtttgtcggtgtgtattgtaaactgagtggcgaattgttcatctaagtcttg   900
agtttgttacgttatgtttcaaattacaaaatttctccagatgttctgccttagtctcaa   960
aaaaaaaaaaaaaaaaaa                                             978
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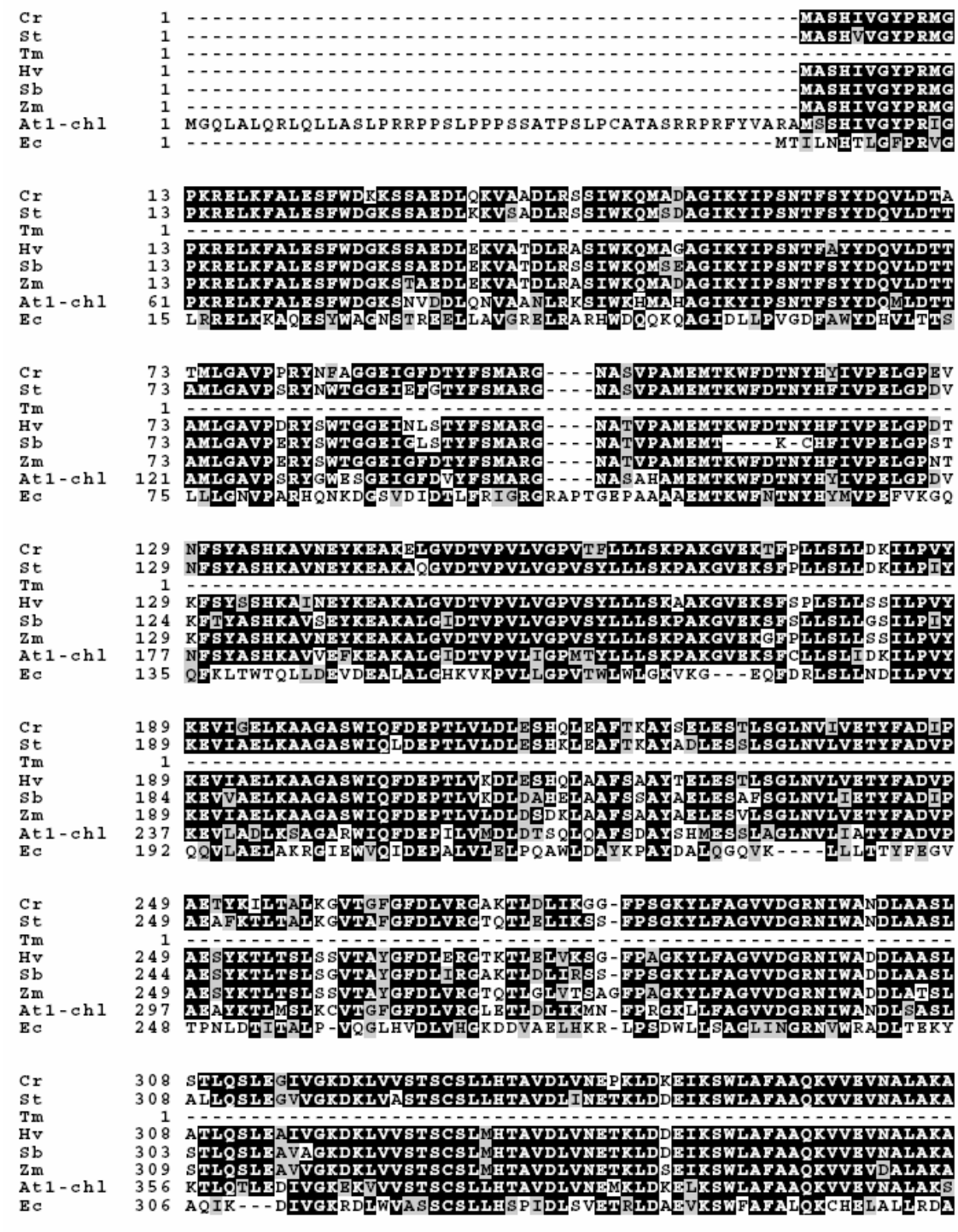
Fig. 4.4 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-Met Syn. Numbers in the right margin refer to the base pair and amino acid positions. Stop codon is in *bold*. A conserved motif WVNPDCGLKTR is highlighted by a *grey* background. The cysteine residue in this motif is *bold italics*.


Cr 1 ----------MASHIVGYPRMG
St 1 ----------MASHVVGYPRMG
Tm 1 ----------
Hv 1 ----------MASHIVGYPRMG
Sb 1 ----------MASHIVGYPRMG
Zm 1 ----------MASHIVGYPRMG
At1-chl 1 MGQLALQRLQLLASLPRRPPSLPPPSSATPSLPCATASRRPRFYVARAMSSHIVGYPRIG
Ec 1 ----------MTILNHTLGFPRVG
Cr 13 PKRELKFALESFWDKKSSAEDLQKVAADLRSSIWKQMADAGIKYIPSNTFSYYDQVLDTA
St 13 PKRELKFALESFWDGKSSAEDLKKVSADLRSSIWKQMSDAGIKYIPSNTFSYYDQVLDTT
Tm 1 ----------
Hv 13 PKRELKFALESFWDGKSSAEDLEKVATDLRASIWKQMAGAGIKYIPSNTFAYYDQVLDTT
Sb 13 PKRELKFALESFWDGKSSAEDLEKVATDLRSSIWKQMSEAGIKYIPSNTFSYYDQVLDTT
Zm 13 PKRELKFALESFWDGKSTAEDLEKVATDLRASIWKQMADAGIKYIPSNTFSYYDQVLDTT
At1-chl 61 PKRELKFALESFWDGKSNVDDLQNVAANLRKSIWKHMAHAGIKYIPSNTFSYYDQMLDTT
Ec 15 LRRELKKAQESYWAGNSTREELLAVGRELRARHWDQQKQAGIDLLPVGDFAWYDHVLTTS
Cr 73 TMLGAVPPRYNFAGGEIGFDTYFSMARG----NASVPAMEMTKWFDTNYHYIVPELGPEV
St 73 AMLGAVPSRYNWTGGEIEFGTYFSMARG----NASVPAMEMTKWFDTNYHFIVPELGPDV
Tm 1 ----------
Hv 73 AMLGAVPDRYSWTGGEINLSTYFSMARG----NATVPAMEMTKWFDTNYHFIVPELGPDT
Sb 73 AMLGAVPERYSWTGGEIGLSTYFSMARG----NATVPAMEMT----K-CHFIVPELGPST
Zm 73 AMLGAVPERYSWTGGEIGFDTYFSMARG----NATVPAMEMTKWFDTNYHFIVPELGPNT
At1-chl 121 AMLGAVPSRYGWESGEIGFDVYFSMARG----NASAHAMEMTKWFDTNYHYIVPELGPDV
Ec 75 LLLGNVPARHQNKDGSVDIDTLFRIGRGRAPTGEPAAAAEMTKWFNTNYEYMVPEFVKGQ
Cr 129 NFSYASHKAVNEYKEAKELGVDTVPVLVGPVTFLLLSKPAKGVEKTFPLLSLFDKILPVY
St 129 NFSYASHKAVNEYKEAKAQGVDTVPVLVGPVSYLLLSKPAKGVEKSFPLLSLFDKILPIY
Tm 1 ----------
Hv 129 KFSYSSHKAINEYKEAKALGVDTVPVLVGPVSYLLLSKAAKGVEKSFSPLSLFSSILPVY
Sb 124 KFTYASHKAVSEYKEAKALGIDTVPVLVGPVSYLLLSKPAKGVEKSFSLLSLFGSILPIY
Zm 129 KFSYASHKAVNEYKEAKALGVDTVPVLVGPVSYLLLSKPAKGVEKGFPLLSLFSSILPVY
At1-chl 177 NFSYASHKAVVEFKEAKALGIDTVPVLIGPMTYLLLSKPAKGVEKSFCLLSLIDKILPVY
Ec 135 QFKLTWTQLLDEVDEALALGHKVKPVLLGPVTWLWLGKVKG---EQFDRLSLFNDILPVY
Cr 189 KEVIGELKAAGASWIQFDEPTLVLDLESHQLEAFTKAYSELESTLSGLNVIVETYFADIP
St 189 KEVIAELKAAGASWIQLDEPTLVLDLESHKLEAFTKAYADLESSLSGLNVLVETYFADVP
Tm 1 ----------
Hv 189 KEVIAELKAAGASWIQFDEPTLVKDLESHQLAAFSAAYTELESTLSGLNVLVETYFADVP
Sb 184 KEVVAELKAAGASWIQFDEPTLVKDLDAHELAAFSSAYAELESAFSGLNVLIETYFADIP
Zm 189 KEVIAELKAAGASWIQFDEPTLVLDLDSDKLAAFSAAYAELESVLSGLNVLVETYFADVP
At1-chl 237 KEVLADLKSAGARWIQFDEPILVMDLDTSQLQAFSDAYSHMESSLAGLNVLIATYFADVP
Ec 192 QQVLAELAKRGIEWVQIDEPALVIELPQAWLDAYKPAYDALQGQVK----LLLTTYFEGV
Cr 249 AETYKILTALKGVTGFGFDLVRGAKTLDLIKGG-FPSGKYLFAGVVDGRNIWANDLAASL
St 249 AEAFKTLTALKGVTAFGFDLVRGTQTLELIKSS-FPSGKYLFAGVVDGRNIWANDLAASL
Tm 1 ----------
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Sb 244 AESYKTLTSLSGVTAYGFDLIRGAKTLDLIRSS-FPSGKYLFAGVVDGRNIWADDLAASL
Zm 249 AESYKTLTSLSSVTAYGFDLVRGTQTLGLVTSAGFPAGKYLFAGVVDGRNIWADDLATSL
At1-chl 297 AEAYKTLMSLKCVTGFGFDLVRGLETLDLIKMN-FPRGKLLFAGVVDGRNIWANDLSASL
Ec 248 TPNLDTITALP-VQGLHVDLVHGKDDVAELHKR-LPSDWLLSAGLINGRNVWRADLTEKY
Cr 308 STLQSLEGLVGKDKLVVSTSCSLLHTAVDLVNEPKLDKEIKSWLAFAAQKVVEVNALAKA
St 308 ALLQSLEGVVGKDKLVASTSCSLLHTAVDLINETKLDDEIKSWLAFAAQKVVEVNALAKA
Tm 1 ----------
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Sb 303 STLQSLEAVAGKDKLVVSTSCSLMHTAVDLVNETKLDDEIKSWLAFAAQKVVEVNALAKA
Zm 309 STLQSLEAVVGKDKLVVSTSCSLMHTAVDLVNETKLDSEIKSWLAFAAQKVVEVDALAKA
At1-chl 356 KTLQTLEDIVGKEKVVVSTSCSLLHTAVDLVNEMKLDKELKSWLAFAAQKVVEVNALAKS
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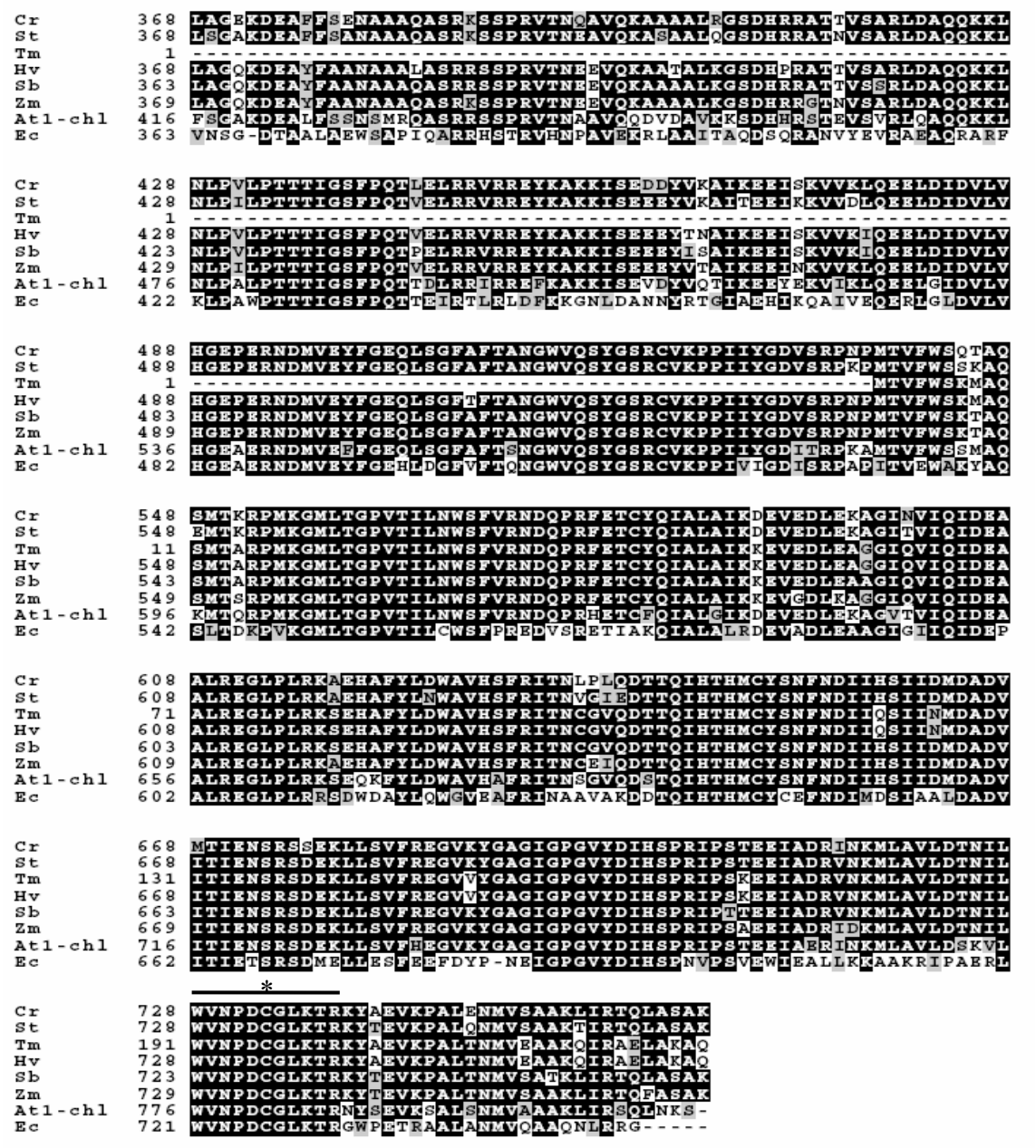

Fig. 4.5 Alignment of the deduced amino acid sequences of Met Syns. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Hv, *Hordeum vulgare* (BAD34660); Sb, *Sorghum bicolor* (AAL73979); Zm, *Zea mays* (AAL33589); St, *Solanum tuberosum* (AAF74983, Zeh et al., 2002); Cr, *Catharanthus* roseus (CAA58474, Eichel et al., 1995); At1, *Arabidopsis thaliana* (CAE55865, Ravanel et al., 2004); Ec, *Escherichia coli* (AAA23544, González et al., 1992). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. The *black bar* indicates the conserved C-terminal motif. *Asterisk* indicate cysteine residue in this motif. At1-chl contains an N-termianl signal peptide which targets to chloroplast.

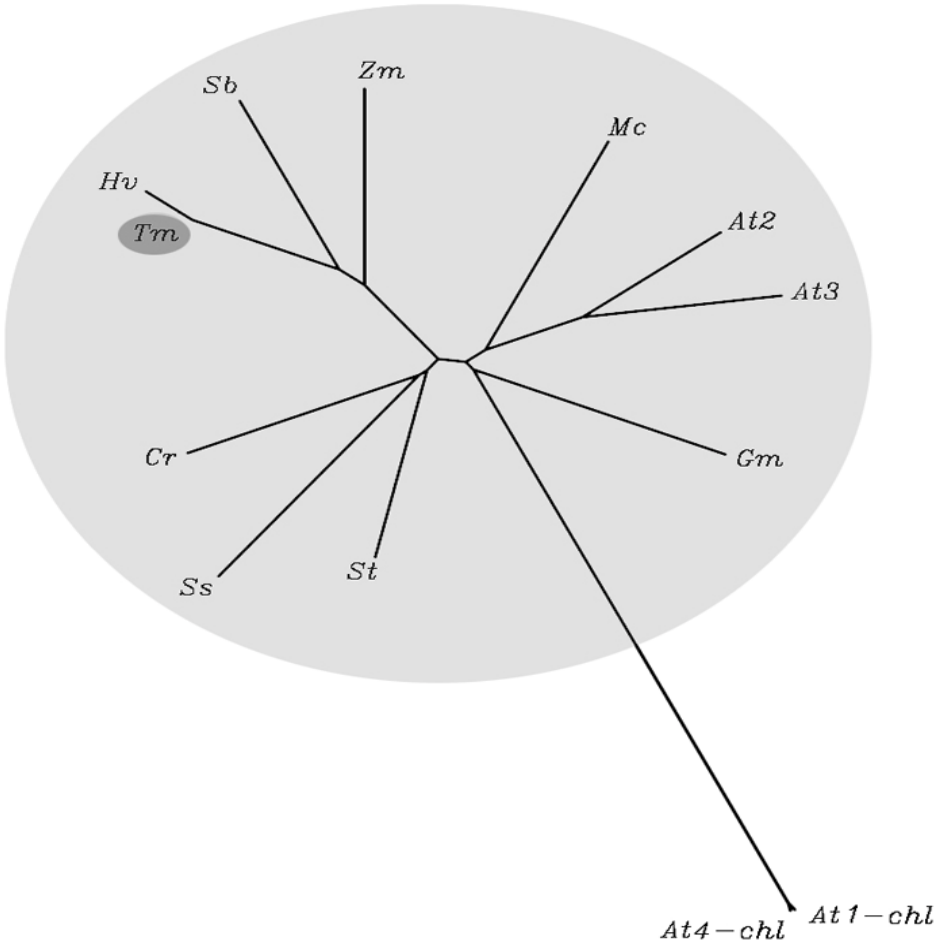

Fig. 4.6 Phylogenetic tree of plant Met Syns. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Hv, *Hordeum vulgare* (BAD34660); Sb, *Sorghum bicolor* (AAL73979); Zm, *Zea mays* (AAL33589); Cr, *Catharanthus* roseus (CAA58474, Eichel et al., 1995); Ss, *Solenostemon scutellarioides* (Q42662); St, *Solanum tuberosum* (AAF74983, Zeh et al., 2002); Mc, *Mesembryanthemum crystallinum* (AAB418969); Gm, *Glycine* max (AAQ08403); At1, 2, 3 and 4, *Arabidopsis thaliana* (CAE55865, CAE55863, CAE55864 (Ravanel et al., 2004), NP_197598, respectively). There are two clusters demonstrated here, one is cytosolic Met Syns (shade in *light grey*), the other is chloroplastic (*chl*) Met Syns.

4.2.3 S-adenosylmethionine synthetase (AdoMet Syn2661 and AdoMet Syn605)

A full-length cDNA clone 441E-2661 was identified and designated *Tm-AdoMet Syn2661*. The clone was 1570 bp in length and had an ORF of 1182 bp. *Tm-AdoMet Syn2661* encodes a protein of 394 amino acids (Fig. 4.7) with a predicted molecular mass of 42.8 KDa and a theoretical pI of 5.61. The coding region was flanked by an 89 bp untranslated region at the 5' end and 299 bp at the 3' end. A conserved amino acid sequence motif was identified in this protein as the hexapeptide GAGDQG (position 121-126), a putative ATP binding site followed by a Lys residue 19 amino acids downstream. The second motif is a glycine-rich signature peptide GGGAFSGKD in position 268 - 276 (Fig. 4.7).

A partial cDNA clone 441E-605 was isolated and named *Tm-AdoMet Syn605*. Like the AdoMet Syn2661 protein, there is also a putative ATP binding site and a signature peptide (Fig. 4.8).

The predicted amino acid sequence of Tm-AdoMet Syn2661 and Tm-AdoMet Syn605 showed 96.2% and 97.2% identity respectively to the *Hordeum vulgare AdoMet Syn* (Hv, GenBank accession number P50299), *Oryza sativa1* (91.9% & 90.8%) (Os1, AAT94053), *Litchi chinensis* (92.1% & 90.8%) (Lc, AAP13994), *Populus deltoides* (91.6% & 89.8%) (Pd, P47916, Van Doorsselaere et al., 1993), *Catharanthus roseus1* (90.8% & 89.1%) (Cr1, CAA95856, Schröder et al., 1997), *Catharanthus roseus2* (91.9% & 89.8%) (Cr2, CAA95857, Schröder et al., 1997), *Catharanthus roseus3* (85.4% & 82.7%) (Cr3, CAA95858, Schröder et al., 1997), *Actinidia chinensis1* (85.6% & 83.8%) (Ac1, AAA81377, Whittaker et al., 1995), *Actinidia chinensis2* (84.9% & 83.5%) (Ac2, AAA81378, Whittaker et al., 1995), *Petunia hybrida* (84.9% & 82.4%)

(Ph, CAA57696, Izhaki et al., 1995) and *Lycopersicon esculentum3* (86.4% & 84.5%) (Le3, CAA80867, Espartero et al., 1994) (Fig. 4.9). The sequence alignment showed that the two AdoMet Syns were closely related. Tm-AdoMet Syn2661 showed 96.8% identity to Tm-AdoMet Syn605 (Fig. 4.9).

A relationship phylogenetic tree of the AdoMet Syn proteins form various plants indicated that the enzymes clustered into two main groups called type I and type II (Fig. 4.10). Both Tm-AdoMet Syn2661 and Tm-AdoMet Syn605 belong to type I enzyme. A more detailed comparison of the two types of AdoMet Syns showed that the differences are often distinguished by characteristic amino acid exchanges at specific positions (Fig. 4.9, asterisks).

The subcellular localization predicted by using ChloroP, MITOPROT, TargetP (Emanuelsson et al., 2000) and PSORT showed that the predicted amino acid sequences of AdoMet Syns are absent of a detectable signal, suggesting that Tm-AdoMet Syn2661 and Tm-AdoMet Syn605 may locate in the cytoplasm.

```
cctcgtgccgattcggcacgagggcggcggcagcagcagcggcagcgcaagaggtagat -28
tggagtatcactaccaccactcaagaaatggcggccgagactttcctcttcacctctgag    33
                          M  A  A  E  T  F  L  F  T  S  E      11
tctgtgaacgagggccaccctgacaagctctgcgaccaggtctctgacgctgtgcttgac    93
S  V  N  E  G  H  P  D  K  L  C  D  Q  V  S  D  A  V  L  D      31
gcatgcctcgcccaagacgctgacagcaaggttgcctgtgagacatgcaccaagaccaac   153
A  C  L  A  Q  D  A  D  S  K  V  A  C  E  T  C  T  K  T  N      51
atggtcatggtttttggtgagatcaccaccaaggccactgttgactatgagaagattgtg   213
M  V  M  V  F  G  E  I  T  T  K  A  T  V  D  Y  E  K  I  V      71
cgtgacacctgccgcaacattggcttcatctctgatgatgttggccttgatgctgaccgg   273
R  D  T  C  R  N  I  G  F  I  S  D  D  V  G  L  D  A  D  R      91
tgcaaggtgcttgtcaacatagagcagcagtctcctgacattgcccagggtgtgcacgga   333
C  K  V  L  V  N  I  E  Q  Q  S  P  D  I  A  Q  G  V  H  G     111
catttcaccaagcgccctgaggatattggtgctggtgaccagggtatcatgtttggctat   393
H  F  T  K  R  P  E  D  I  G  A  G  D  Q  G  I  M  F  G  Y     131
gccaccgatgagacccctgagctcatgcccctcagccatgtgcttgccaccaagctgggt   453
A  T  D  E  T  P  E  L  M  P  L  S  H  V  L  A  T  K  L  G     151
gcccgcctcactgaagtccgcaagaatggcacttgtgcctggctaaggcctgatggcaaa   513
A  R  L  T  E  V  R  K  N  G  T  C  A  W  L  R  P  D  G  K     171
acccaggtcacggttgaatacctcaacgagggaggtgccatggttcctgtacgtgtgcac   573
T  Q  V  T  V  E  Y  L  N  E  G  G  A  M  V  P  V  R  V  H     191
actgttctgatctccacccagcatgatgagactgtcaccaatgatgagattgctgcggac   633
T  V  L  I  S  T  Q  H  D  E  T  V  T  N  D  E  I  A  A  D     211
ctcaaggagcatgtcatcaagccggttatccctgagaagtacctggacgagaagaccata   693
L  K  E  H  V  I  K  P  V  I  P  E  K  Y  L  D  E  K  T  I     231
ttccacttgaatccatcaggccgctttgtcattggtggccctcatggtgatgctggtctt   753
F  H  L  N  P  S  G  R  F  V  I  G  G  P  H  G  D  A  G  L     251
actggtcgcaagatcatcatcgacacctatggtggctggggagcacacggaggtggtgct   813
T  G  R  K  I  I  I  D  T  Y  G  G  W  G  A  H  G  G  G  A     271
ttctctggcaaggacccaactaaggttgaccgcagtggcgcctacattgccaggcaggct   873
F  S  G  K  D  P  T  K  V  D  R  S  G  A  Y  I  A  R  Q  A     291
gccaagagcatcattgctagcggtcttgcacgccgctgcattgttcagatctcctacgcc   933
A  K  S  I  I  A  S  G  L  A  R  R  C  I  V  Q  I  S  Y  A     311
attggtgtgcctgagcccttatctgtctttgttgactcttatggtactggcaagatcccc   993
I  G  V  P  E  P  L  S  V  F  V  D  S  Y  G  T  G  K  I  P     331
gataaggagatcctcaagattgtgaaggagaactttgacttcaggcctgggatgatcagc  1053
D  K  E  I  L  K  I  V  K  E  N  F  D  F  R  P  G  M  I  S     351
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I  N  L  D  L  K  K  G  G  N  R  F  I  K  T  A  A  Y  G  H     371
tttggccgtgaggatgctgacttcacctgggaggttgtgaagcccctcaagtttgacaag  1173
F  G  R  E  D  A  D  F  T  W  E  V  V  K  P  L  K  F  D  K     391
gcctctgcttgagaggtgggtatgaaggagtctatcattcctgctgctgccgctggtggt  1233
A  S  A  stop                                                   394
tgtatcttggataattgaattccttctctttagaaggaagcagggtttcaacatcgccgt  1293
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gagttaatactagtcgggtcttttgatgtcttctgtgctttgattttgctttttgagtct  1413
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gcatctgtaaaaaaaaaaaaaaaaaaa                                    1500
```

Fig. 4.7 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-AdoMet Syn2661. Numbers in the right margin refer to the base pair and amino acid positions. Start and stop codons are in *bold*. The conserved ATP-binding site (GAGDQG) and signature peptide (GGGAFSGKD) are highlighted by a *grey* background. A Lys residue 19 amino acids downstream of GAGDQG is shown in *bold italics*.

```
ggacacttcaccaagcgtcccgaagaggtcggcgccggtgaccagggcatcatgttcggc     60
G  H  F  T  K  R  P  E  E  V  G  A  G  D  Q  G  I  M  F  G      20
tatgccaccgacgagacccctgagctgatgcccctcagccacgtgcttgccaccaagctc    120
Y  A  T  D  E  T  P  E  L  M  P  L  S  H  V  L  A  T  K  L      40
ggagctcgcctcaccgaggtccgcaagaatggcacctgcgcctgggttaggcctgacgga    180
G  A  R  L  T  E  V  R  K  N  G  T  C  A  W  V  R  P  D  G      60
aagacccaggtcaccgtcgagtacctaaacgaggatggtgccatggtacctgttcgtgtg    240
K  T  Q  V  T  V  E  Y  L  N  E  D  G  A  M  V  P  V  R  V      80
cacaccgtcctcatctccacccagcacgacgagaccgtcaccaacgacgagattgccgcg    300
H  T  V  L  I  S  T  Q  H  D  E  T  V  T  N  D  E  I  A  A     100
gacctcaaggagcatgtcatcaagccggtgatccccgcgaagtacctcgatgagaacacc    360
D  L  K  E  H  V  I  K  P  V  I  P  A  K  Y  L  D  E  N  T     120
atcttccacctgaacccgtctggccgcttcgtcatcggcggccctcacggtgacgccggt    420
I  F  H  L  N  P  S  G  R  F  V  I  G  G  P  H  G  D  A  G     140
ctcactggccgcaagatcatcatcgacacctatggtggctggggagcccacggcggcggt    480
L  T  G  R  K  I  I  I  D  T  Y  G  G  W  G  A  H  G  G  G     160
gccttctctggcaaggacccaaccaaggtcgaccgcagtggcgcctacattgccaggcag    540
A  F  S  G  K  D  P  T  K  V  D  R  S  G  A  Y  I  A  R  Q     180
gccgccaagagcatcattgccagcggcctcgcacgccgctgcattgtgcagatctcatac    600
A  A  K  S  I  I  A  S  G  L  A  R  R  C  I  V  Q  I  S  Y     200
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cccgacagggagatcctcaagctcgtgaaggagaactttgacttcaggcccgggatgatc    720
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K  A  S  A  STOP                                                284
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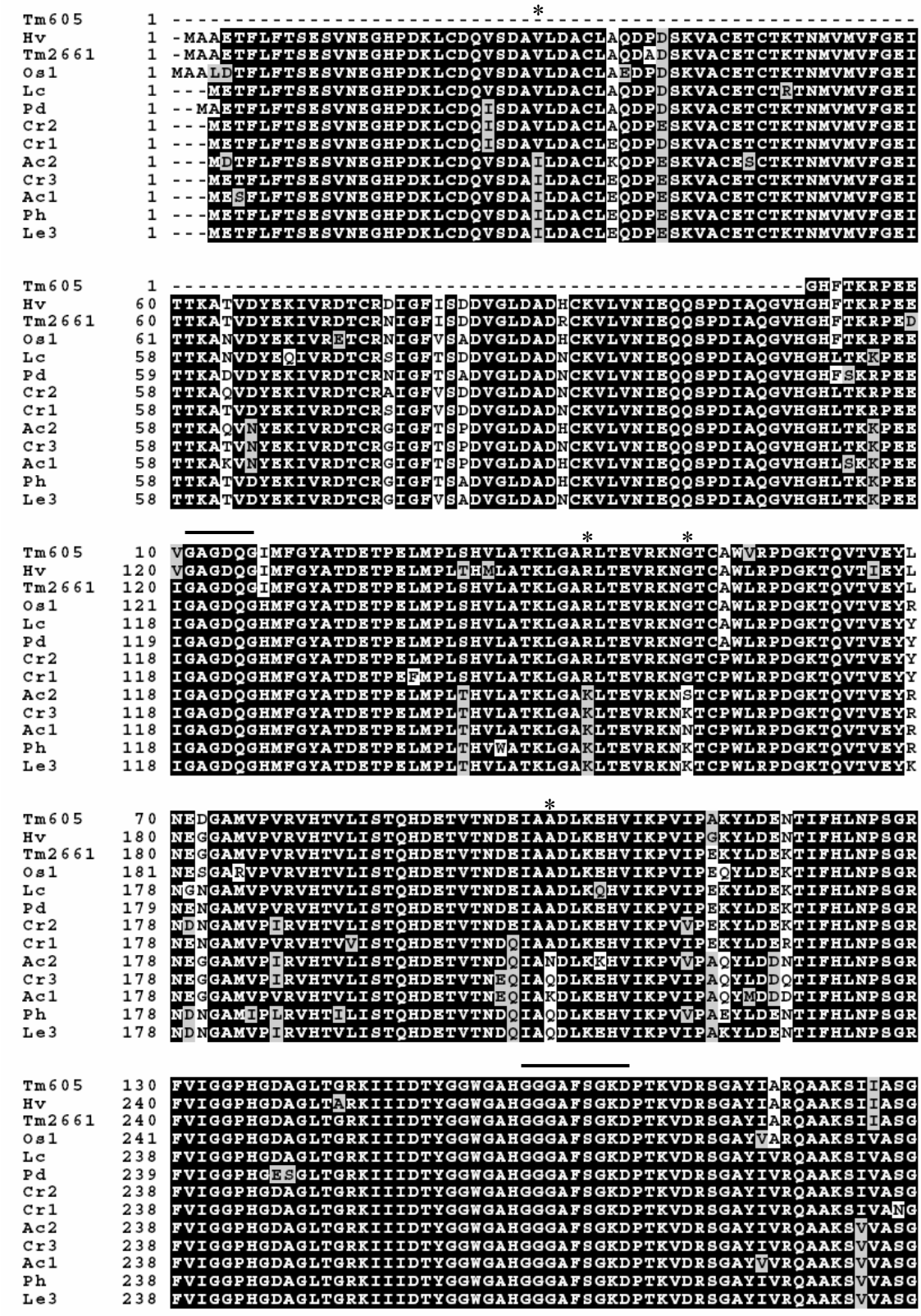
gagtcttggaaagataataaagagcgatgtgtaattcaaaaaaaaaaaaaaaaaaaa      1197
```

Fig. 4.8 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-AdoMet Syn605. Numbers in the right margin refer to the base pair and amino acid positions. Stop codon is in *bold*. The conserved ATP-binding site (GAGDQG) and signature peptide (GGGAFSGKD) are highlighted by a *grey* background. A Lys residue 19 amino acids downstream of GAGDQG is shown in *bold italics*.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Os1 1 MAALDTFLFTSESVNEGHPDKLCDQVSDAVLDACLAEDPDSKVACETCTKTNMVMVFGEI
Lc 1 ---METFLFTSESVNEGHPDKLCDQVSDAVLDACLAQDPDSKVACETCTRTNMVMVFGEI
Pd 1 --MAETFLFTSESVNEGHPDKLCDQISDAVLDACLAQDPDSKVACETCTKTNMVMVFGEI
Cr2 1 ---METFLFTSESVNEGHPDKLCDQISDAVLDACLAQDPESKVACETCTKTNMVMVFGEI
Cr1 1 ---METFLFTSESVNEGHPDKLCDQISDAVLDACLEQDPDSKVACETCTKTNMVMVFGEI
Ac2 1 ---MDTFLFTSESVNEGHPDKLCDQVSDAILDACLKQDPESKVACESCTKTNMVMVFGEI
Cr3 1 ---METFLFTSESVNEGHPDKLCDQVSDAILDACLEQDPESKVACETCTKTNMVMVFGEI
Ac1 1 ---MESFLFTSESVNEGHPDKLCDQVSDAILDACLEQDPESKVACETCTKTNMVMVFGEI
Ph 1 ---METFLFTSESVNEGHPDKLCDQVSDAILDACLEQDPESKVACETCTKTNMVMVFGEI
Le3 1 ---METFLFTSESVNEGHPDKLCDQVSDAILDACLEQDPESKVACETCTKTNMVMVFGEI
Tm605 1 ----------GHFTKRPEE
Hv 60 TTKATVDYEKIVRDTCRDIGFISDDVGLDADHCKVLVNIEQQSPDIAQGVHGHFTKRPEE
Tm2661 60 TTKATVDYEKIVRDTCRNIGFISDDVGLDADRCKVLVNIEQQSPDIAQGVHGHFTKRPED
Os1 61 TTKANVDYEKIVRETCRNIGFVSADVGLDADHCKVLVNIEQQSPDIAQGVHGHFTKRPEE
Lc 58 TTKANVDYEQIVRDTCRSIGFTSDDVGLDADNCKVLVNIEQQSPDIAQGVHGHLTKKPEE
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Cr1 58 TTKATVDYEKIVRDTCRSIGFVSDDVGLDADNCKVLVNIEQQSPDIAQGVHGHLTKRPEE
Ac2 58 TTKAQVNYEKIVRDTCRGIGFTSPDVGLDADHCKVLVNIEQQSPDIAQGVHGHLTKKPEE
Cr3 58 TTKATVNYEKIVRDTCRGIGFTSPDVGLDADNCKVLVNIEQQSPDIAQGVHGHLTKKPEE
Ac1 58 TTKAKVNYEKIVRDTCRGIGFTSPDVGLDADHCKVLVNIEQQSPDIAQGVHGHLSKKPEE
Ph 58 TTKATVDYEKIVRDTCRGIGFTSADVGLDADHCKVLVNIEQQSPDIAQGVHGHLTKKPEE
Le3 58 TTKATVDYEKIVRDTCRGIGFVSADVGLDADNCKVLVNIEQQSPDIAQGVHGHLTKKPEE
* *
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Hv 120 VGAGDQGIMFGYATDETPELMPLTHMLATKLGARLTEVRKNGTCAWLRPDGKTQVTIEYL
Tm2661 120 IGAGDQGIMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEYL
Os1 121 IGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEYR
Lc 118 IGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEYY
Pd 119 IGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEYY
Cr2 118 IGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCPWLRPDGKTQVTVEYY
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Cr3 118 IGAGDQGHMFGYATDETPELMPLTHVLATKLGAKLTEVRKNKTCPWLRPDGKTQVTVEYR
Ac1 118 IGAGDQGHMFGYATDETPELMPLTHVLATKLGAKLTEVRKNNTCPWLRPDGKTQVTVEYR
Ph 118 IGAGDQGHMFGYATDETPELMPLTHVWATKLGAKLTEVRKNKTCPWLRPDGKTQVTVEYR
Le3 118 IGAGDQGHMFGYATDETPELMPLTHVLATKLGAKLTEVRKNKTCPWLRPDGKTQVTVEYK
*
Tm605 70 NEDGAMVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPAKYLDENTIFHLNPSGR
Hv 180 NEGGAMVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPGKYLDENTIFHLNPSGR
Tm2661 180 NEGGAMVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDEKTIFHLNPSGR
Os1 181 NESGARVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEQYLDEKTIFHLNPSGR
Lc 178 NGNGAMVPVRVHTVLISTQHDETVTNDEIAADLKQHVIKPVIPEKYLDEKTIFHLNPSGR
Pd 179 NENGAMVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDEKTIFHLNPSGR
Cr2 178 NDNGAMVPIRVHTVLISTQHDETVTNDEIAADLKEHVIKPVVPEKYLDEKTIFHLNPSGR
Cr1 178 NENGAMVPVRVHTVVISTQHDETVTNDQIAADLKEHVIKPVIPEKYLDERTIFHLNPSGR
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Ac1 178 NEGGAMVPVRVHTVLISTQHDETVTNEQIAKDLKEHVIKPVIPAQYMDDDTIFHLNPSGR
Ph 178 NDNGAMIPLRVHTILISTQHDETVTNDQIAQDLKEHVIKPVVPAEYLDENTIFHLNPSGR
Le3 178 NDNGAMVPIRVHTVLISTQHDETVTNDQIAQDLKEHVIKPVIPAKYLDENTIFHLNPSGR
Tm605 130 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIIASG
Hv 240 FVIGGPHGDAGLTARKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIIASG
Tm2661 240 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIIASG
Os1 241 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYVARQAAKSIVASG
Lc 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSIVASG
Pd 239 FVIGGPHGESGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSIVASG
Cr2 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSIVASG
Cr1 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSIVANG
Ac2 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSVVASG
Cr3 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSVVASG
Ac1 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYVVRQAAKSVVASG
Ph 238 FVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIVRQAAKSVVASG
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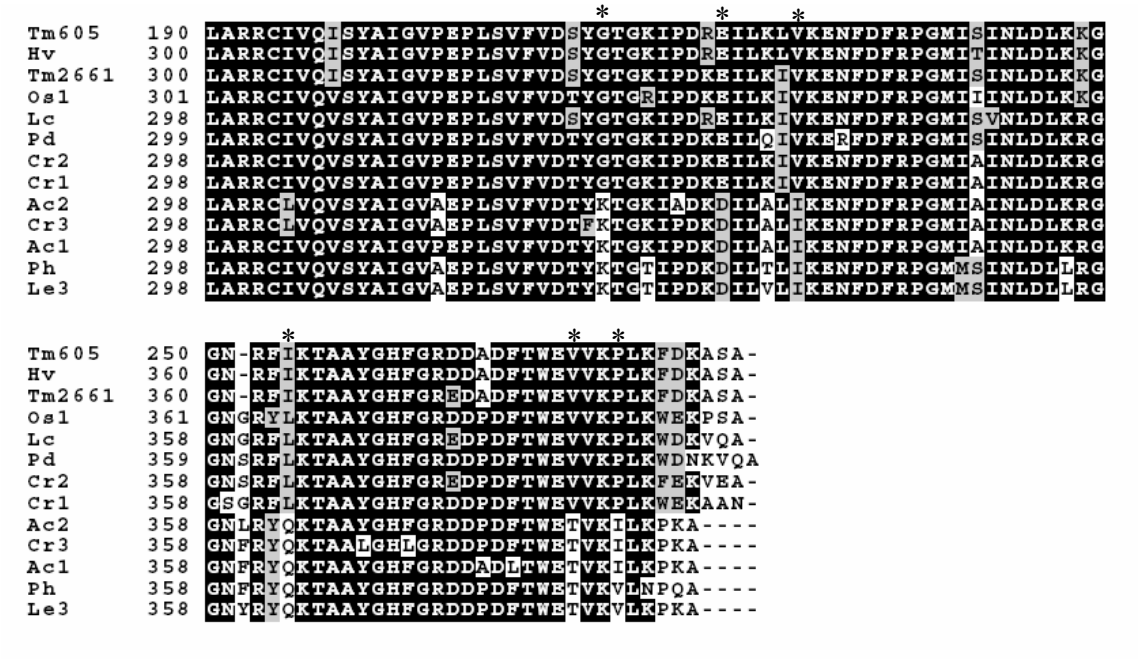

Fig. 4.9 Alignment of the deduced amino acid sequences of AdoMet Syns from various plants. The GenBank accession numbers or references: Tm605 and Tm2661, *Triticum monococcum*; Hv, *Hordeum vulgare* (P50299); Os1, *Oryza sativa* (AAT94053); Lc, *Litchi chinensis* (AAP13994); Pd, *Populus deltoids* (P47916, Van Doorsselaere et al., 1993); Cr1, 2 and 3, *Catharanthus roseus* (CAA95856, CAA95857, CAA95858, respectively, Schröder et al., 1997); Ac1 and 2, *Actinidia chinensis* (AAA81377, AAA81378 respectively, Whittaker et al., 1995); Ph, *Petunia hybrida* (CAA57696, Izhaki et al., 1995); Le3, *Lycopersicon esculentum* (CAA80867, Espartero et al., 1994). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. *Asterisks* indicate the positions in which type I and type II proteins contain consistent differences. The conserved signature peptides are indicated in *black bars*.

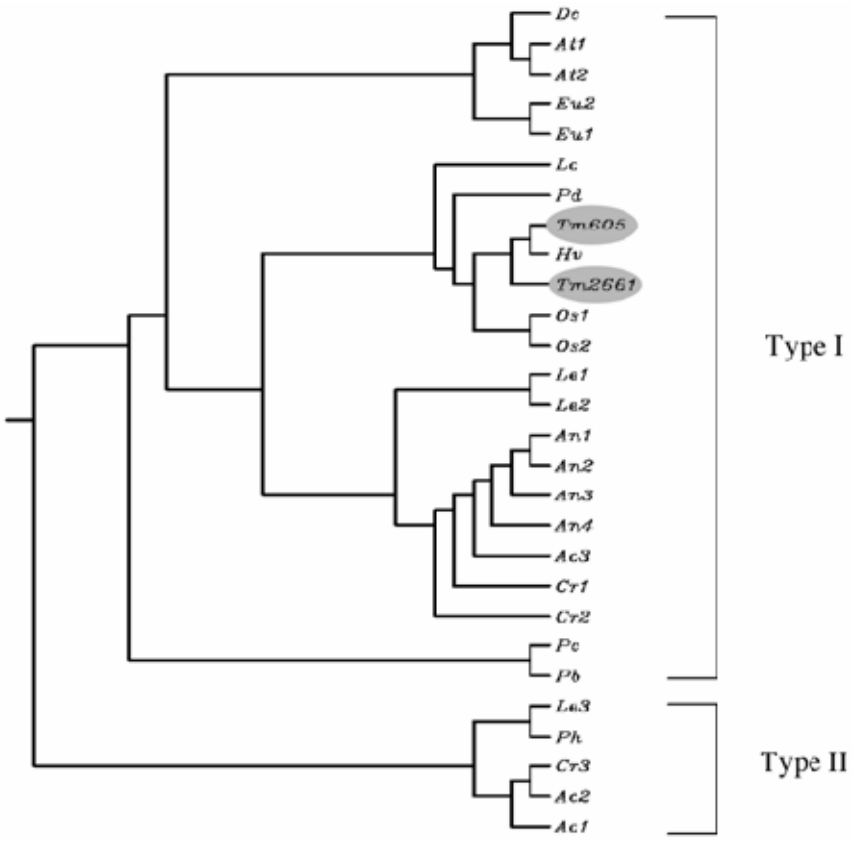

Fig. 4.10 Phylogenetic tree of plant AdoMet Syns. The GenBank accession numbers or references: Tm605 and Tm2661, *Triticum monococcum*; Hv, *Hordeum vulgare* (P50299); Os1 and 2, *Oryza sativa* (AAT94053, CAC82203, respectively); Lc, *Litchi chinensis* (AAP13994); Pd, *Populus deltoids* (P47916, Van Doorsselaere et al., 1993); Le1, 2 and 3, *Lycopersicon esculentum* (CAA80865, CAA80866, CAA80867, respectively, Espartero et al., 1994); An1, 2, 3 and 4, *Atriplex nummularia* (BAD29707, BAD29708, BAD29710, BAD29711, respectively); Cr1, 2 and 3, *Catharanthus roseus* (CAA95856, CAA95857, CAA95858, respectively, Schröder et al., 1997); Dc, *Dendrobium crumenatum* (AAL16064); At1 and 2, *Arabidopsis thaliana* (AAA32868, AAA32869, respectively, Peleman et al., 1989a, 1989b); Eu1 and 2, *Elaeagnus umbellate* (AAK29409, AAK29410, respectively); Pc, *Pinus contorta* (AAG17036, Lindroth et al., 2001); Pb, *Pinus banksiana* (P50300); Ph, *Petunia hybrida* (CAA57696, Izhaki et al., 1995); Ac1, 2 and 3, *Actinidia chinensis* (AAA81377, AAA81378, AAA81379, respectively, Whittaker et al., 1995).

4.2.4 S-adenosylmethionine decarboxylase (SAMDC)

A cDNA clone 441E-886 encoding a putative SAMDC, designated *Tm-SAMDC*, obtained from the 441E-cDNA library, following BLAST screening, was fully sequenced. The total cDNA has 1945 bp and contains an ORF of 1164 bp coding for a protein of 388 amino acids in length. The *SAMDC* transcript contained an unusually long 5'-untranslated region of 517 nucleotides and a 3' noncoding region of 264 nucleotides (Fig. 4.11). The predicted SAMDC protein has an estimated molecular weight of 42.4 KDa and theoretical pI of 4.98. A conserved region LSESS can be found in positon 68 - 72 and a putative cleavage site of the SAMDC pro-enzyme is indicated by a triangle (Fig. 4.11). The cleavage of SAMDC proenzyme, resulting in the formation of a small β-chain in the N-terminus and a larger α-chain in the C-terminus, was confirmed to be essential for enzyme activity. The second highly conserved region is TIHVTPEDGFSYASYE which is a putative PEST sequence (Fig. 4.11). The 5' leader of the mRNA contained start and stop codons for a polypeptide of 49 amino acids, and this region was conserved in the 5' leaders of other plant SAMDC mRNAs (Fig. 4.11, asterisks).

Comparison of the predicted amino acid sequences of SAMDC from various plant species showed that Tm-SAMDC has high similarity to several other plant genes, including *Triticum aestivum* (97.2%) (Ta, GenBank accession number AAD17232, Li and Chen, 2000a), *Oryza sativa1* (84.0%) (Os1, AAC79990, Li and Chen, 2000b), *Zea mays* (81.2%) (Zm, CAA69075, Franceschetti et al., 2001), *Catharanthus roseus* (52.1%) (Cr, Q42679, Schröeder and Schröeder, 1995), *Pisum sativum* (51.3%) (Ps, AAB03865, Marco and Carrasco, 2002) and *Spinacia oleracea* (50.1%) (So, CAA57170, Bolle et al.,

1995) (Fig. 4.12). The alignment indicated that the monocot enzymes had an extended C-terminus relative to dicot and human (Fig. 4.12). The monocot enzymes were about 20 residues longer than that of the dicot enzymes.

Phylogenetic analysis was used to compare the amino acid sequences of Tm-SAMDC with those from other plants (Fig. 4.13). The results indicated that Tm-SAMDC has high degree of identity to monocots *T. aestivum* (97.2%), *O. sativa1* (84.0%), *Z. mays* (81.2%) and low degree of identity to dicot *S. oleracea* (50.1%).

The subcellular localization predicted by using ChloroP, MITOPROT, TargetP (Emanuelsson et al., 2000) and PSORT showed that the predicted amino acid sequence of *SAMDC* contained no transit peptide for chloroplastic or mitochondrial localization, suggesting that *SAMDC* may encode a cytosolic SAMDC.

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cctcccacccccaccgtctcgccgccgccaccaccgccgcagctagataagaagaaaaaag -457
agagaagaactcgtcgggagggctcgagatctgtgtcgggagaggggaattcttgagat  -397
cggaatcggaacaagcgggcgcgctcgggatcggggttaccatacaatttttcccaggaa  -337
cctttagtgaatgtgctaatggagtcaaagggtggcaaaaagtctagcagtagtagttcc  -277
                  M  E  S  K  G  G  K  K  S  S  S  S  S  S
                  *  *  *  *  *  *  *  *  *  *  *  *     *
ctgatgtacgaagctcccctcggctacagcattgaagacgttcgacctgctggaggcgcc  -217
L  M  Y  E  A  P  L  G  Y  S  I  E  D  V  R  P  A  G  G  A
      *  *  *  *  *  *  *  *  *  *  *  *  *  *     *
aagaagttctctgctgcatactcgaactgcgcgaagaagccatcctgatatcgtttttgg  -157
K  K  F  S  A  A  Y  S  N  C  A  K  K  P  S  stop
*  *  *  *  *  *  *  *  *  *     *  *  *
ctccccttcccgtagtttaggatttttatgcaatttattctgactcttttctcccacc   -97
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tcctctctgctctgctctctgactcgaactgcaacaatggctgccccgacctctgcgatc    24
                                    M  A  A  P  T  S  A  I      8
gggtttgagggctacgagaagcgcctcgagatcaccttctccgaggcatcaatctttgcc    84
G  F  E  G  Y  E  K  R  L  E  I  T  F  S  E  A  S  I  F  A     28
gaccctcatggtcgtggcctgcgcgccctctccagggcccagattgactctgttcttgat   144
D  P  H  G  R  G  L  R  A  L  S  R  A  Q  I  D  S  V  L  D     48
cttgcacggtgcaccattgtgtccgagctctccaacaaggacttcgactcctatgtgctc   204
L  A  R  C  T  I  V  S  E  L  S  N  K  D  F  D  S  Y  V  L     68
tctgagtcgagcctgttcatctactcccagaagattgtgatcaagacctgtgggactacc   264
S  E  S  S  L  F  I  Y  S  Q  K  I  V  I  K  T  C  G  T  T     88
      ▲
atgctcctgctcaccattcctaggattcttgagcttgctgaagagctgtgcatgccgctt   324
M  L  L  L  T  I  P  R  I  L  E  L  A  E  E  L  C  M  P  L    108
gctgccgtgaagtactctcgtgggatgttcatcttccctggcgcgcagcctgctccccac   384
A  A  V  K  Y  S  R  G  M  F  I  F  P  G  A  Q  P  A  P  H    128
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```
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ggcaatgcttatgtgatcggagacccagcgaagcctggccagaagtggcacatctactat   504
G  N  A  Y  V  I  G  D  P  A  K  P  G  Q  K  W  H  I  Y  Y     168
gccaccgagcaacctgagcagcccatggtcaccctggagatgtgcatgactgggctggac   564
A  T  E  Q  P  E  Q  P  M  V  T  L  E  M  C  M  T  G  L  D     188
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K  K  K  A  S  V  F  F  K  T  Q  A  D  G  H  V  S  C  A  K     208
gagatgaccaagctctctggtatctccgacatcattcccgagatggaggtctgcgacttt   684
E  M  T  K  L  S  G  I  S  D  I  I  P  E  M  E  V  C  D  F     228
gactttgagccctgcggctactccatgaacgccatcaacggatctgccgtctccaccatc   744
D  F  E  P  C  G  Y  S  M  N  A  I  N  G  S  A  V  S  T  I     248
catgtgacccccgaggacggcttcagctatgcgagctacgaggtcatgggcatggacgcc   804
H  V  T  P  E  D  G  F  S  Y  A  S  Y  E  V  M  G  M  D  A     268
tctgccctggcctacggcgacatcgtcaagagggtcctccggtgctttggcccttcagag   864
S  A  L  A  Y  G  D  I  V  K  R  V  L  R  C  F  G  P  S  E     288
ttctctgtggcggtcaccatctttggtggccgcggccacgccgccacctggggcaagaag   924
F  S  V  A  V  T  I  F  G  G  R  G  H  A  A  T  W  G  K  K     308
ctcgacgccgaggcatacgactgcaacaacgttgtggagcaggagctaccctgcggcggc   984
L  D  A  E  A  Y  D  C  N  N  V  V  E  Q  E  L  P  C  G  G     328
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V  L  V  Y  Q  S  F  T  A  N  E  E  V  A  V  S  A  G  S  P     348
aggtccgtcttccactgcttcgaggccgagagtgtgcagagccaccctctggtcaaggaa  1104
R  S  V  F  H  C  F  E  A  E  S  V  Q  S  H  P  L  V  K  E     368
ggcaagcttgccaacctcctcgcatggcgggcggaggaggaggacgcggtgctgtgcgag  1164
G  K  L  A  N  L  L  A  W  R  A  E  E  E  D  A  V  L  C  E     388
tgatgcgataatctgctgtctctgttccgtctgtggaatttctttgactgttgtcgtttg  1224
stop
tcgtttggttactgtgaagcagccggccaggctattgctctctgaataaactattagctc  1284
taggtggtttgctgcgtctgccacaatgagcatactgtttggcctcgtgccgaattcggc  1344
acgagggtcaaaggcaactaacaggatgaggtctgtactgattctttgcgttattattgc  1404
tgctgctgtggcatgcccattatcaaaaaaaaaa                            1438
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Fig. 4.11 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-SAMDC. Numbers in the right margin refer to the base pair and amino acid positions. Start and stop codons are in *bold.* The conserved proenzyme cleavage site and PEST domains are highlighted by *grey* background, and the *black triangle* indicates the cleavage site. Conserved residues in the 5' leader of plant SAMDC mRNAs are labeled in *asterisks.*

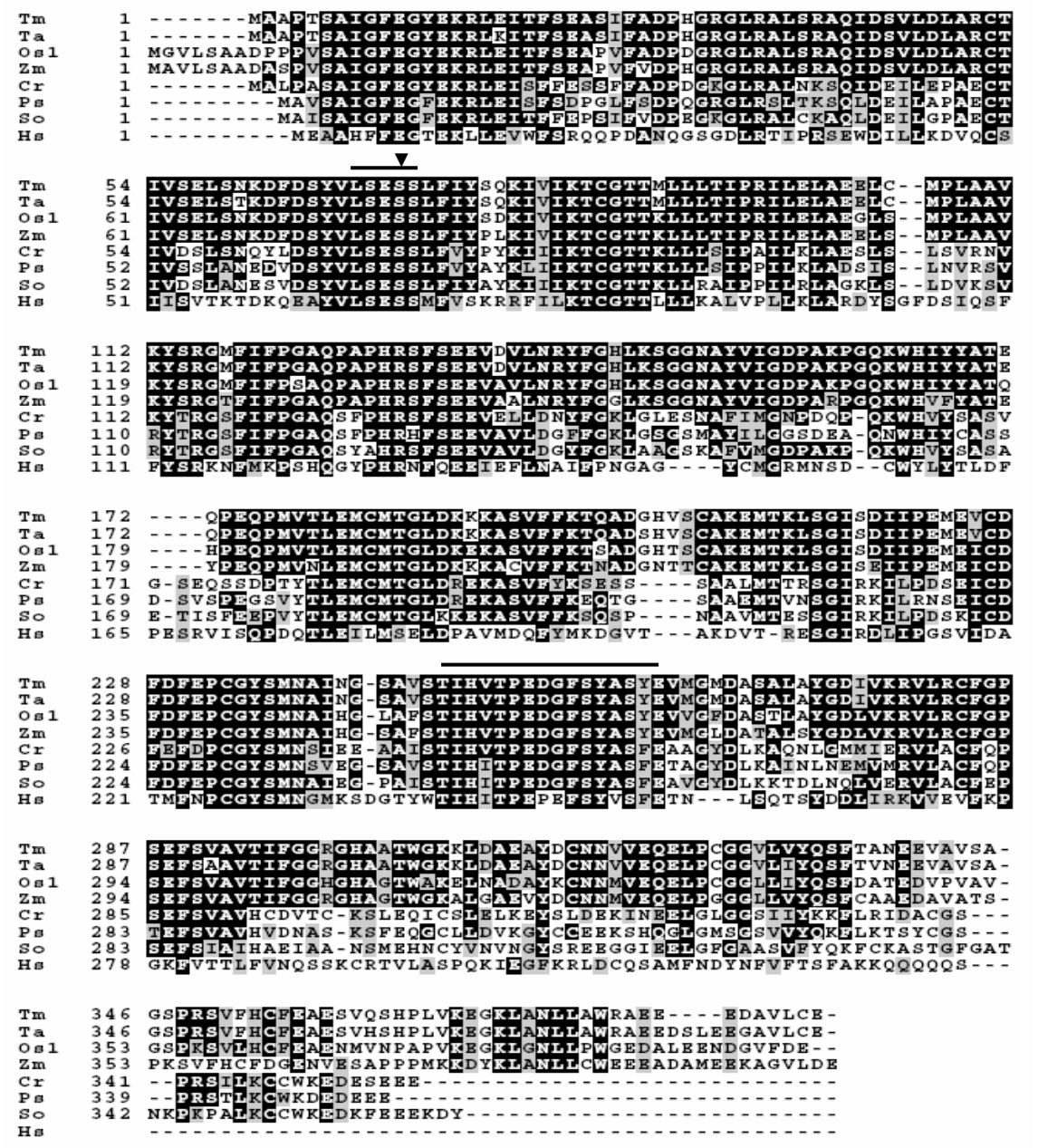

Fig. 4.12 Alignment of the deduced amino acid sequences of SAMDCs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Ta, *Triticum aestivum* (AAD17232, Li and Chen, 2000a); Os1, *Oryza sativa* (AAC79990, Li and Chen, 2000b); Zm, *Zea mays* (CAA69075, Franceschetti et al., 2001); Cr, *Catharanthus roseus* (Q42679, Schröeder and Schröeder, 1995); Ps, *Pisum sativum* (AAB03865, Marco and Carrasco, 2002); So, *Spinacia oleracea* (CAA57170, Bolle et al., 1995); Hs, *Homo sapiens* (AAA51716, Pajunen et al., 1988). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. Two conserved regions LSESS and TIHVTPEDGFSYASYE are indicated in *black bar*. The *black triangle* indicates the cleavage site.

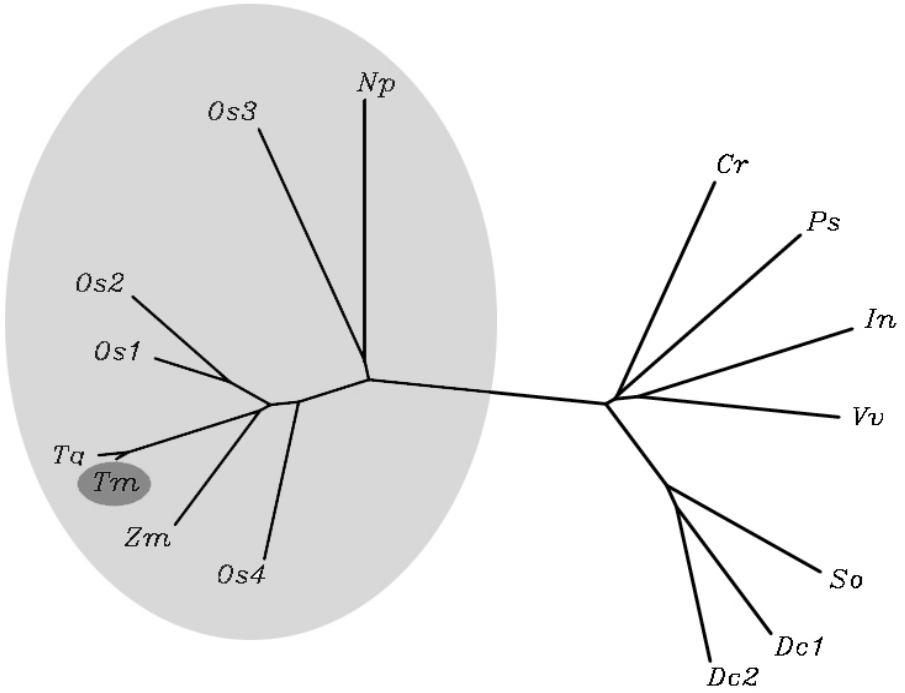

Fig. 4.13 Phylogenetic tree of plant SAMDCs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Ta, *Triticum aestivum* (AAD17232, Li and Chen, 2000a); Zm, *Zea mays* (CAA69075, Franceschetti et al., 2001); Os1, 2, 3 and 4, *Oryza sativa* (AAC79990 (Li and Chen, 2000b), CAC09522, BAD33432, CAB64600 (Franceschetti et al., 2001), respectively); Np, *Narcissus pseudonarcissus* (AAO43186); Cr, *Catharanthus roseus* (Q42679, Schröder and Schröder, 1995); Ps, *Pisum sativum* (AAB03865, Marco and Carrasco, 2002); In, *Ipomoea nil* (Q96471); Vv, *Vitis vinifera* (CAD98785); So, *Spinacia oleracea* (CAA57170, Bolle et al., 1995); Dc1 and 2, *Dianthus caryophyllus* (AAD09839, AAD09840, respectively, Lee et al., 1997). There are two clusters demonstrated here, one is monocot SAMDCs (shade in *light grey*), the other is dicot SAMDCs.

4.2.5 Serine hydroxymethyltransferase (SHMT)

A cDNA clone, designated *Tm-SHMT* and containing an 1811 bp insert, was sequenced and found to contain one ORF of 1530 nucleotides encoding a protein of 510 amino acids. The coding region was flanked at the 5' end by 69 bp of noncoding sequence and at the 3' end, 212 bp (Fig. 4.14). The calculated molecular weight was 56.1 KDa and the predicated pI, 8.18. A pyridoxal-phosphate attachment site DVVTTTTHKSLRGPRGA was found in position 271-287 in wheat sequence (Fig. 4.14, grey background). A mitochondrial target signal peptide MAMATALRKLSARGQPLSRL was found at the N-terminal end (Fig. 4.14, underline).

Sequence analysis of Tm-SHMT, in combination with a computer-assisted search in the available data banks, revealed large similarity with DNA encoding SHMT from various organisms. The Tm-SHMT has 93.1%, 85.1%, 84.5% and 56.7% similarity at the protein level with the SHMT from *Oryza sativa1* (Os1-chl, GenBank accession number AAP44712), *Pisum sativum* (Ps-mit, P34899, Turner et al., 1992), *Solanum tuberosum* (St-mit, CAA81082, Kopriva and Bauwe, 1995) and *Homo sapiens* (Hs, AAA63257, Garrow et al., 1993), respectively (Fig. 4.15). SHMT is a pyridoxal-phosphate containing enzyme. The pyridoxal-P group is attached to a lysine residue around which the sequence is highly conserved in all forms of the enzyme (Fig. 4.15, black bar).

The phylogenetic tree analysis was used to compare the amino acid sequences of Tm-SHMT with others (Fig. 4.16). The tree demonstrated that Tm-SHMT was grouped into mitochondrial SHMT, rather than chloroplastic or cytosolic SHMT.

Furthermore, the subcellular localization predicted by using ChloroP, MITOPROT, TargetP (Emanuelsson et al., 2000) and PSORT showed that the predicted amino acid sequence of SHMT contained transit peptides for mitochondrial localization, suggesting that *SHMT* may encode a mitochondrial SHMT.

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         M  A  M  A  T  A  L  R  K  L  S  A  R  G  Q  P  L      17

tcccgcctcacgccgctctactccatggcgtccctgccggcgacggaggagagatccgca   111
S  R  L  T  P  L  Y  S  M  A  S  L  P  A  T  E  E  R  S  A      37

gtcacctggccgaagcagttgaacgcgccgctggaggaggtcgaccccgagattgccgac   171
V  T  W  P  K  Q  L  N  A  P  L  E  E  V  D  P  E  I  A  D      57
atcatcgagctcgagaaggcccgccaatggaaggggctggagctcatcccgtcggagaac   231
I  I  E  L  E  K  A  R  Q  W  K  G  L  E  L  I  P  S  E  N      77
ttcacctccctgtcggtgatgcaggcggtggggtccgtcatgaccaacaagtacagcgag   291
F  T  S  L  S  V  M  Q  A  V  G  S  V  M  T  N  K  Y  S  E      97
gggtaccccggcgcgagatactacggtggaaacgaatacatcgatatggccgagacgctg   351
G  Y  P  G  A  R  Y  Y  G  G  N  E  Y  I  D  M  A  E  T  L     117
tgccagaaacgtgctttggaggcctttaatttggacccggagaagtggggagtgaatgtg   411
C  Q  K  R  A  L  E  A  F  N  L  D  P  E  K  W  G  V  N  V     137
caacccctatcgggttcacctgccaacttccatgtatacactgctctgctgaagccacat   471
Q  P  L  S  G  S  P  A  N  F  H  V  Y  T  A  L  L  K  P  H     157
gatagaattatggctctggatcttcctcacggtggacatctttcccatggttaccagact   531
D  R  I  M  A  L  D  L  P  H  G  G  H  L  S  H  G   Y  Q  T     177
gacacaaagaaaatctcagcagtttcaatattctttgagacaatgccttacagactggat   591
D  T  K  K  I  S  A  V  S  I  F  F  E  T  M  P  Y  R  L  D     197
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K  L  I  V  A  G  A  S  A  Y  A  R  L  Y  D  Y  N  R  M  R     237
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F  P  G  L  Q  G  G  P  H  N  H  T  I  T  G  L  A  V  A  L     337
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K  Q  A  T  T  Q  E  Y  R  A  Y  Q  E  Q  V  M  S  N  S  A     357
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R  F  A  E  S  L  T  S  K  G  Y  D  I  V  S  G  G  T  D  N     377
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L  E  N  V  H  I  A  A  N  K  N  T  V  P  G  D  V  S  A  M     417
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D  F  A  K  V  A  D  F  F  D  S  A  V  N  L  A  L  K  V  K     457
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A  A  A  A  G  T  K  L  K  D  F  V  A  T  L  Q  S  D  S  N     477
atccaagctgaaattgcaaagcttcgccacgatgtggaggaatatgcgaaacaattccca  1491
I  Q  A  E  I  A  K  L  R  H  D  V  E  E  Y  A  K  Q  F  P     497
acaattggattcgagaaagagaccatgaagtacaagaactaagaactgctatgtttcaac  1551
T  I  G  F  E  K  E  T  M  K  Y  K  N  stop                    510
agcaaggaagcaaacaagaagcacagctgaggacaagtccatgtaaacaatagatccatg  1611
atgaaggccatcttatgtaaaaggaatccaagcattttacagaatatgggaactttgtca  1671
atagtttcttattgcaggcacatactgtaagatgcttcgctgatatgctatatgaactgc  1731
catccttggataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa    1788
```

Fig. 4.14 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-SHMT. Numbers in the right margin refer to the base pair and amino acid positions. Start and stop codons are in *bold.* The *underline* indicates the mitochodria target peptide. SHMT pyridoxal-phosphate attachment site was labeled in *grey* background.

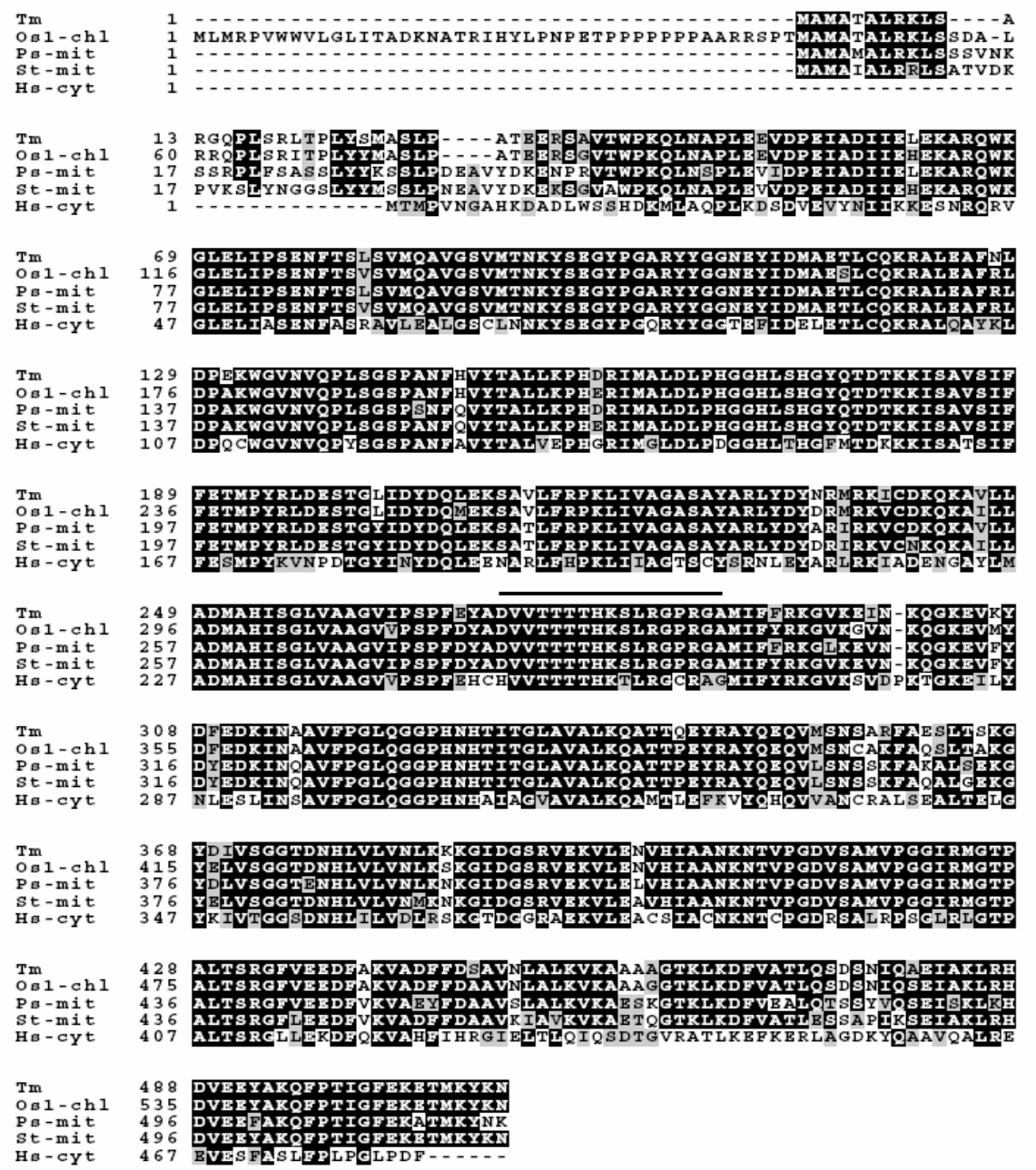

Fig. 4.15 Alignment of the deduced amino acid sequences of SHMTs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Os1, *Oryza sativa* (AAP44712); Ps, *Pisum sativum* (P34899, Turner et al., 1992); St, *Solanum tuberosum* (CAA81082, Kopriva and Bauwe, 1995); Hs, *Homo sapiens* (AAA63257, Garrow et al., 1993). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. The *black bar* indicates the highly conserved sequence in all forms of the enzyme. Os1-chl contains an N-terminal signal peptide which targets to chloroplast. Ps-mit and St-mit contain an N-terminal signal peptide which targets to mitochondria.

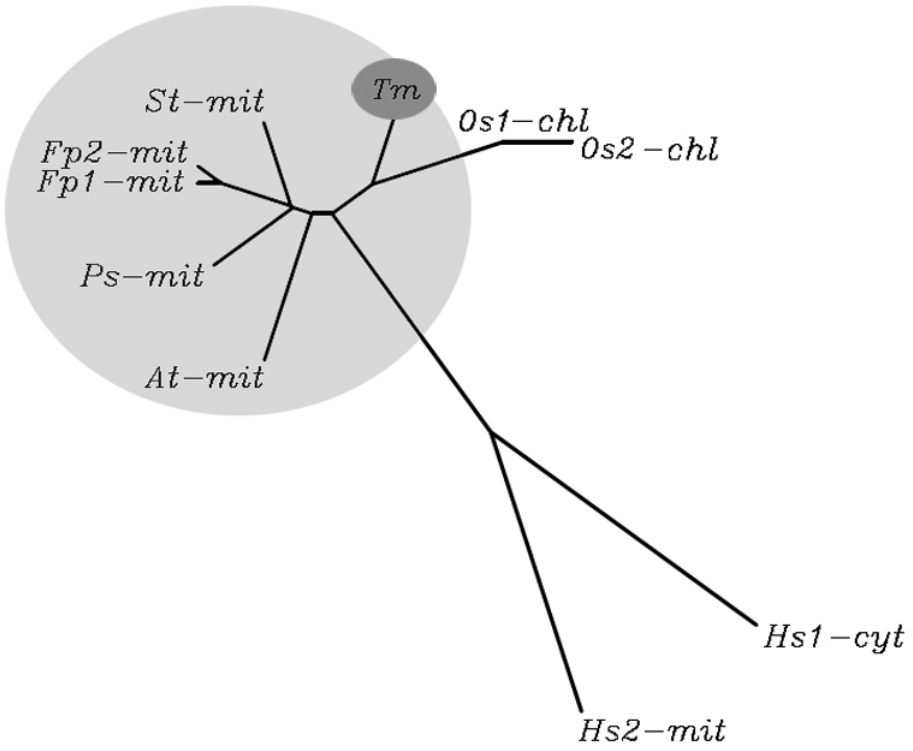

Fig. 4.16 Phylogenetic tree of SHMTs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Os1 and 2, *Oryza sativa* (AAP44712, AAR07090 (Ilic et al., 2003) respectively); Ps, *Pisum sativum* (P34899, Turner et al., 1992); St, *Solanum tuberosum* (CAA81082, Kopriva and Bauwe, 1995); Fp1 and 2, *Flaveria pringlei* (P49357, P49358, respectively); At, *Arabidopsis thaliana* (AAK59622); Hs1 and 2, *Homo sapiens* (AAA63257, AAA63258 (Garrow et al., 1993) respectively). There are three clusters demonstrated here, one is mitochondrial (*mit*) SHMTs (shade in *light grey*), the second is chloroplastic (*chl*) SHMTs, the third is cytosolic (*cyt*) SHMTs.

4.2.6 N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase / N^5, N^{10}-methylenetetrahydrofolate dehydrogenase (THFC/THFD)

A cDNA clone of 1024 bp was sequenced and called *Tm-THFC/THFD*, which had a 5'-untranslated region of 21 bp, a 127 bp noncoding 3' region and an 876 bp coding region encoding a protein of 292 amino acids (Fig. 4.17). The predicted molecular weight was 30.8 KDa and the theoretical pI was 7.77. A highly conserved sequence of 13 amino acids (LTPVPGGVGPMTV) is located at the C-terminal section of position 263- 275 (Fig. 4.17).

The deduced amino acid sequence for Tm-THFC/THFD was aligned using CLUSTALW program sequence analysis software. The results showed that Tm-THFC/THFD shared 88.7%, 78.4%, 51.7%, 41.4%, 46.5% and 16.8% amino acid sequence homology with *Oryza sativa* (Os, GenBank accession number AAG48834), *Pisum sativum* (Ps, T50664), *Arabidopsis thaliana4* (At4-chl, AAM62762), *Mus musculus* (Mm, AAA39827), *Escherichia coli* (Ec, AAA23803) amd *Saccharomyces cerevisiae* (Sc, AAB00323), respectively (Fig. 4.18).

Cluster analysis with the phylogenetic tree program indicated that Tm-THFC/THFD is grouped into cytosolic THFC/THFD, which is quite distant from the mitochondrial and chloroplastic cluster (Fig. 4.19). Furthermore, the subcellular localization predicted by using ChloroP, MITOPROT, TargetP (Emanuelsson et al., 2000) and PSORT showed that the predicted amino acid sequence of THFC/THFD contained no transit peptide for chloroplastic or mitochondrial localization, suggesting that *THFC/THFD* may encode a cytosolic THFC/THFD.

```
agagaatctagtgctacccca atg gcgcaaatcatcgacggcaaggccatcgccggcgaa    39
                      M  A  Q  I  I  D  G  K  A  I  A  G  E     13
atcaggcgcgagatcggcgccgaggtcgccgtgctctcgtccgcccacagcatcgtgccg    99
I  R  R  E  I  G  A  E  V  A  V  L  S  S  A  H  S  I  V  P     33
gggctggcggtggtgatcgtggggagcaggaaggactcgcagacgtacgtgcagatgaag   159
G  L  A  V  V  I  V  G  S  R  K  D  S  Q  T  Y  V  Q  M  K     53
cgcaaggcctgcgccgaggtcggcatccgctccttcgacgtcgacctccccgaggacatc   219
R  K  A  C  A  E  V  G  I  R  S  F  D  V  D  L  P  E  D  I     73
gccgaggccgcgctcgtcgccgaggtccaccgcctcaacgccgaccccgccgtccacgga   279
A  E  A  A  L  V  A  E  V  H  R  L  N  A  D  P  A  V  H  G     93
attcttgttcagcttccattgcccaagcatatcaacgaagaaaatatcttaaaccagatc   339
I  L  V  Q  L  P  L  P  K  H  I  N  E  E  N  I  L  N  Q  I    113
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G  R  D  P  L  F  V  P  C  T  P  K  G  C  M  E  L  L  S  R    153
agtggcgtcactgtaaaaggaaaacacgcagttgtggttgggcgtagcaacatcgtgggt   519
S  G  V  T  V  K  G  K  H  A  V  V  V  G  R  S  N  I  V  G    173
ttaccagtatcccttctccttctgaaagcggacgctaccgtgtcgatcgtgcattcacgg   579
L  P  V  S  L  L  L  L  K  A  D  A  T  V  S  I  V  H  S  R    193
accccaaatcccgaaacaattgtccgtcaagcagacattgtcattgcagcagctggccag   639
T  P  N  P  E  T  I  V  R  Q  A  D  I  V  I  A  A  A  G  Q    213
gccatgatgatcaagggagactggatcaaaccaggcgcggcggtcatcgacgtcgggaca   699
A  M  M  I  K  G  D  W  I  K  P  G  A  A  V  I  D  V  G  T    233
aactccatcgacgacccaaccaggaagtctgggtacagactcgttggcgatgtggatttc   759
N  S  I  D  D  P  T  R  K  S  G  Y  R  L  V  G  D  V  D  F    253
tcggaggcaagcaaggtcgtgggtcacctgactccggtccccggaggcgtcgggccgatg   819
S  E  A  S  K  V  V  G  H  L  T  P  V  P  G  G  V  G  P  M    273
accgtggcgatgttgctgaagaacacggtggacggcgccaagaggggtatagtcagc tga   879
T  V  A  M  L  L  K  N  T  V  D  G  A  K  R  G  I  V  S stop   292
tttgctgcgtgatttggtaatgtggggatgatgttgtaactgggtgcctagactaaaatc   939
ggttccccattttgtcatgtcttacgctatttcgagatcaataaacgacgctcagtcgct   999
caccaaaaaaaaaaaaaaaaa                                         1021
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Fig. 4.17 Nucleotide and deduced amino acid sequences of the cDNA encoding Tm-THFC/THFD. Numbers in the right margin refer to the base pair and amino acid positions. Start and stop codons are in *bold.* A signature peptide (LTPVPGGVGPMTV) is highlighted with a *grey* background.

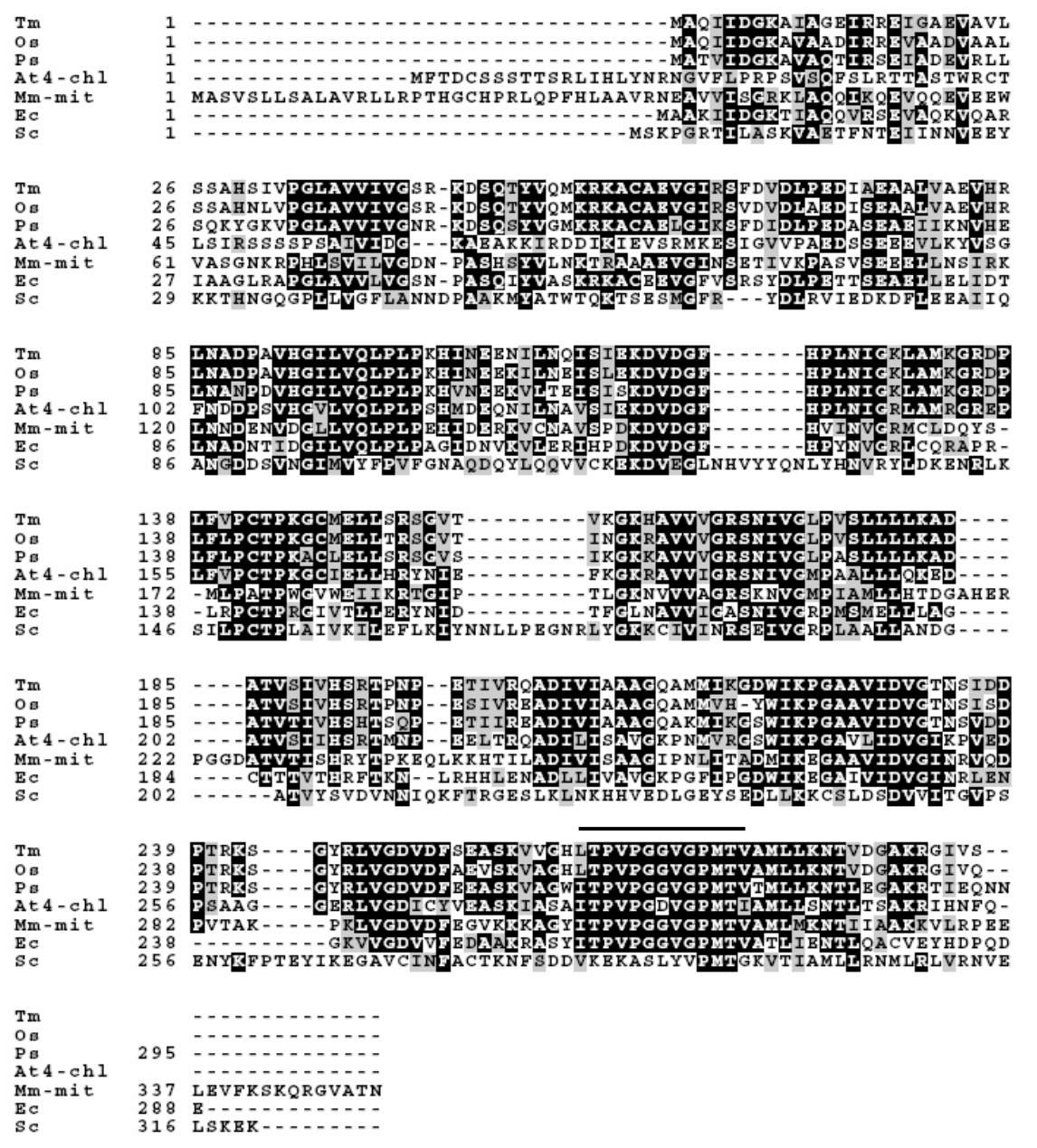

Fig. 4.18 Alignment of the deduced amino acid sequences of THFCs/THFDs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Os, *Oryza sativa* (XP_493923); Ps, *Pisum sativum* (CAB56756); At4, *Arabidopsis thaliana* (CAB80869); Mm, *Mus musculus* (AAA39827, Bélanger and MacKenzie, 1989); Ec, *Escherichia coli* (AAA23803, D'Ari and Rabinowitz, 1991); Sc, *Saccharomyces cerevisiae* (AAB00323, West et al., 1993). Identical residues are highlighted in *black*, similar residues in *grey*. *Dashes* are gaps introduced to maximize alignment. At4-chl contains an N-termianl signal peptide which targets to chloroplast. Mm-mit contains an N-terminal signal peptide which targets to mitochondria. A highly conserved sequence in the bifunctional THFCs/THFDs is indicated in *black bar*.

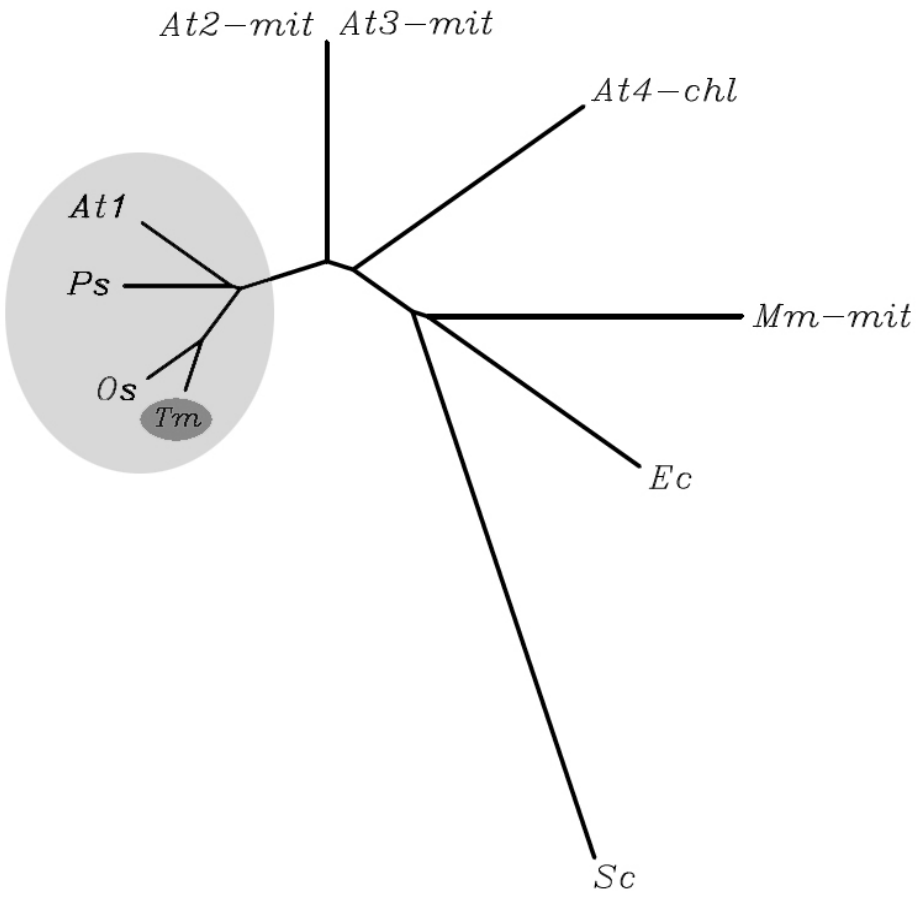

Fig. 4.19 Phylogenetic tree of THFCs/THFDs. The GenBank accession numbers or references: Tm, *Triticum monococcum*; Os, *Oryza sativa* (XP_493923); Ps, *Pisum sativum* (CAB56756); At1, 2, 3 and 4, *Arabidopsis thaliana* (AAM62762, NP_181400, AAC67352, CAB80869, respectively). Mm, *Mus musculus* (AAA39827, Bélanger and MacKenzie, 1989); Ec, *Escherichia coli* (AAA23803, D'Ari and Rabinowitz, 1991); Sc, *Saccharomyces cerevisiae* (AAB00323, West et al., 1993). There are three clusters demonstrated here, one is cytosolic THFCs/THFDs (shade in *light grey*), the second is mitochondrial (*mit*) THFCs/THFDs, the third is chloroplastic (*chl*) THFCs/THFDs.

4.3 Expression of genes in the generation and supply of methyl units in response to wheat powdery mildew infection

To investigate mRNA accumulation of 7 genes involved in the generation and supply of methyl units (Fig. 2.1) in response to biotic stress, 10-day-old susceptible Tm441 and resistant Tm453 were inoculated with wheat powdery mildew *Bgt* and total RNA was extracted from these plants during a 144 hpi time period. Twenty micrograms of total RNA were used for northern blot hybridization. Single bands were detected when hybridized with *MTHFR, Met Syn, AdoMet2661, AdoMet605, SAMDC*, *SHMT* and *THFC/THFD* probes, respectively (Fig. 4.20 and Fig. 4.21).

In the susceptible interaction, *MTHFR* mRNA could be detected at 3 hpi and reached a maximal expression level at 6 hpi. At 12 hpi *MTHFR* expression dipped to background level but then showed a large increase at 24 hpi and 48 hpi before decreasing again through to 144 hpi (Fig. 4.20). The induction patterns of *Met Syn, AdoMet2661, AdoMet605 and SAMDC* were similar to that of *MTHFR*, suggesting the expression of this group of genes is coordinately induced by powdery mildew infection.

The two expression peaks in the susceptible interaction, at 6 hpi and after 24 hpi, match the times in powdery mildew development of primary and appressorial germ tubes, respectively (Fig. 4.20). These two development stages are characterized by the formation of host cell wall appositions and localized H_2O_2 bursts (Thordal-Christensen et al., 1997). However, in the case of the genes, *SHMT and THFC/THFD*, transcript accumulation was down-regulated in Tm441 leaves in response to powdery mildew infection (Fig. 4.20).

Thus we can group these 7 genes into two groups: group I including *MTHFR, Met Syn, AdoMet2661, AdoMet605* and *SAMDC* whose expression is up-regulated; group II including *SHMT* and *THFC/THFD* whose expression is not up-regulated. Although group I and group II genes have different expression patterns in response to powdery mildew infection, both are coordinately expressed at the transcription level (Fig. 4.20).

Compared to the susceptible interaction, the expression pattern of the group I genes in the wheat leaves undergoing resistant interaction was different. In the susceptible interaction, there are two peaks; however, in resistant interaction there is only one at 24 hpi (Fig. 4.21). Expression of group II genes *SHMT and THFC/THFD* is almost constitutively expressed throughout the infection process of wheat powdery mildew, except that *SHMT* transcription level has some slightly increase from 72 hpi until 144 hpi and *THFC/THFD* has slightly increase at 12 hpi and 96 hpi (Fig. 4.21).

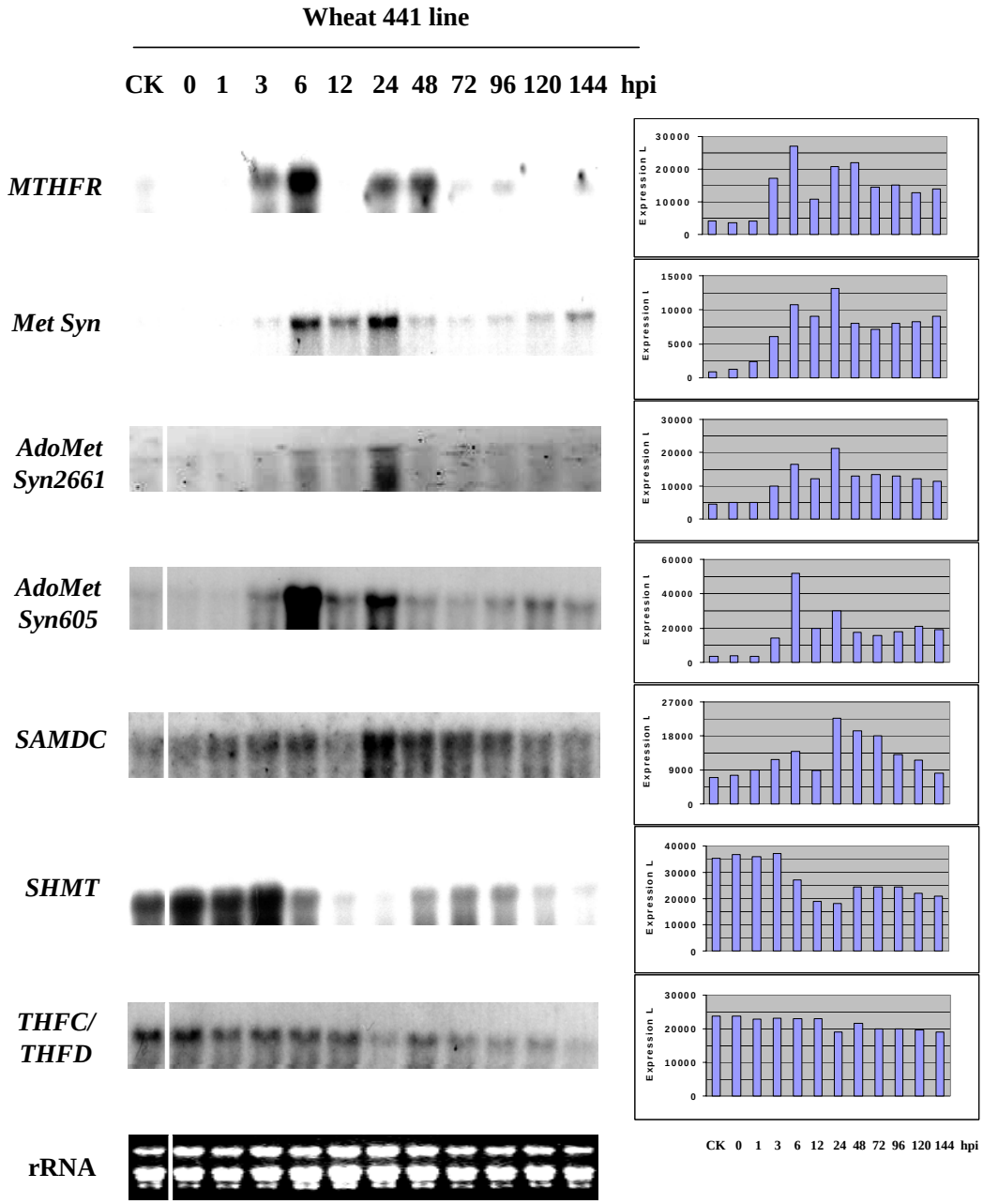

Fig. 4.20 Northern blot analysis of *T. monococum MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605, SAMDC, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 after inoculation with *Blumeria graminis* f. sp. *tritici.* Total RNA was isolated from non-inoculated Tm441 leaves (CK) and leaves at 0, 1, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hour post inoculation (hpi) as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager^{TM} 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

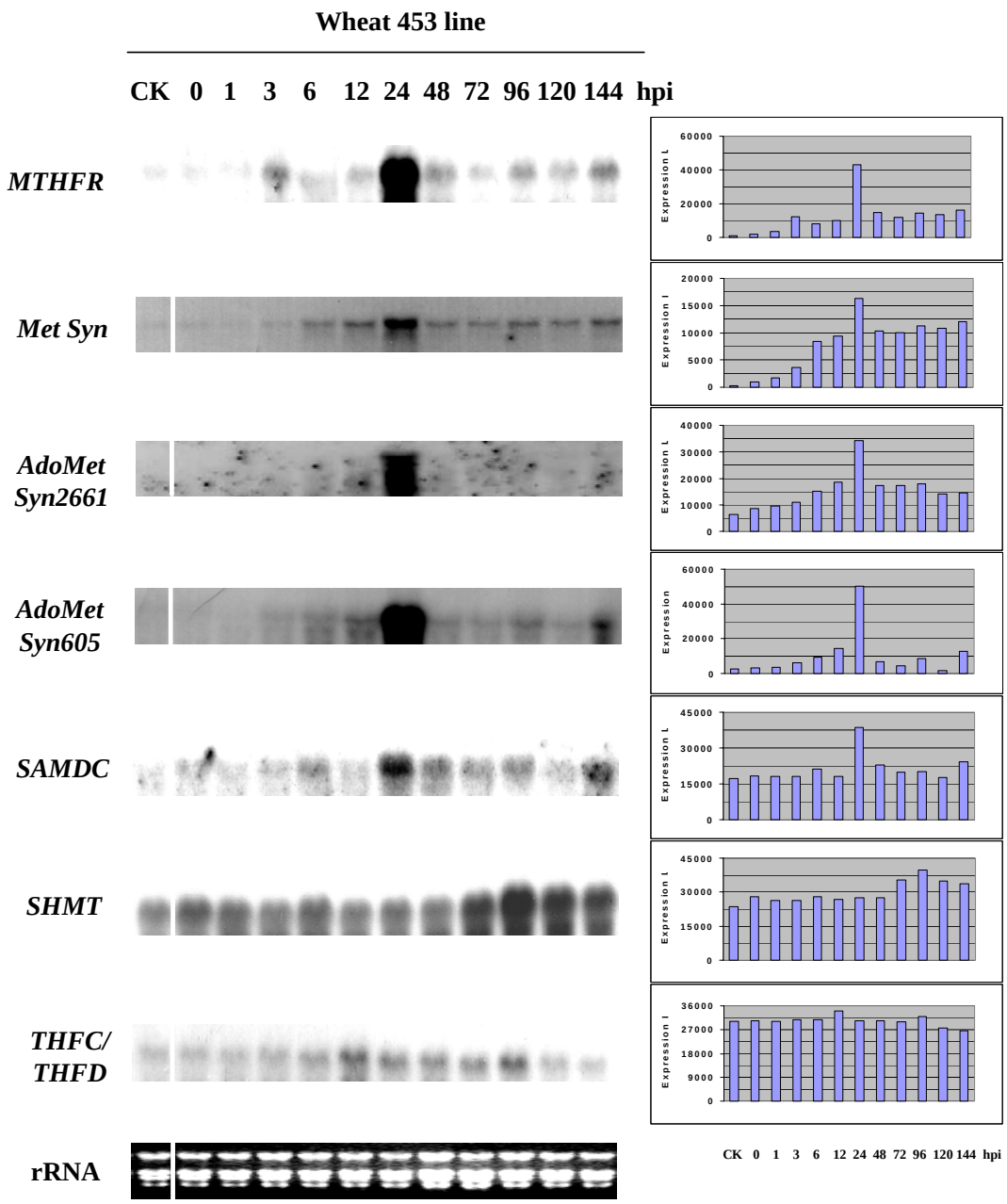

Fig. 4.21 Northern blot analysis of *T. monococcum MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605, SAMDC, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm453 after inoculation with *Blumeria graminis* f. sp. *tritici.* Total RNA was isolated from non-inoculated Tm453 leaves (CK) and leaves at 0, 1, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hour post inoculation (hpi) as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager™ 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.4 Expression of genes in the generation and supply of methyl units in response to abiotic stress treatments

4.4.1 Wounding

To characterize the abiotic stress responsiveness of these 7 genes, total RNA was extracted from leaves of 10-day-old seedlings of diploid Tm441 after wounding. The mRNA level of *MTHFR* was significantly increased at 1 and 6 h, decreased back to the control level at 12 h and then increased again at 24 h (Fig. 4.22). This pattern also occurred with *AdoMet Syn605* and *SAMDC* and is one that is similar to the susceptible interaction, where there were two peaks at 6 and 24 hpi (Fig. 4.20). The other group I gene, *Met Syn*, reached a maximal mRNA level at 6 h, decreasing at 12 h and thereafter (Fig. 4.22). The group II genes, *SHMT and THFC/THFD*, expression almost remained the same throughout (Fig. 4.22).

4.4.2 Drought

Total RNA was harvested from leaves of 10-day-old seedlings of Tm441 after dehydration treatment. Expression of *Met Syn* and *AdoMet Syn605* was coordinately induced by dehydration. Transcripts of *Met Syn* and *AdoMet Syn605* were initially induced at 1 h and peaked at 6 h, following which there was a continuous decline to 24 h (Fig. 4.23). The expression of *MTHFR* mRNA was rapidly induced to the highest level at 1 h after which it decreased sharply (Fig. 4.23). *SAMDC* mRNA was initially induced at 1 h and then increased step by step, reaching its highest level at 24 h (Fig. 4.23). Compared to group I genes, expression of group II genes *SHMT and THFC/THFD* was down-regulated from 12 to 24 h (Fig. 4.23).

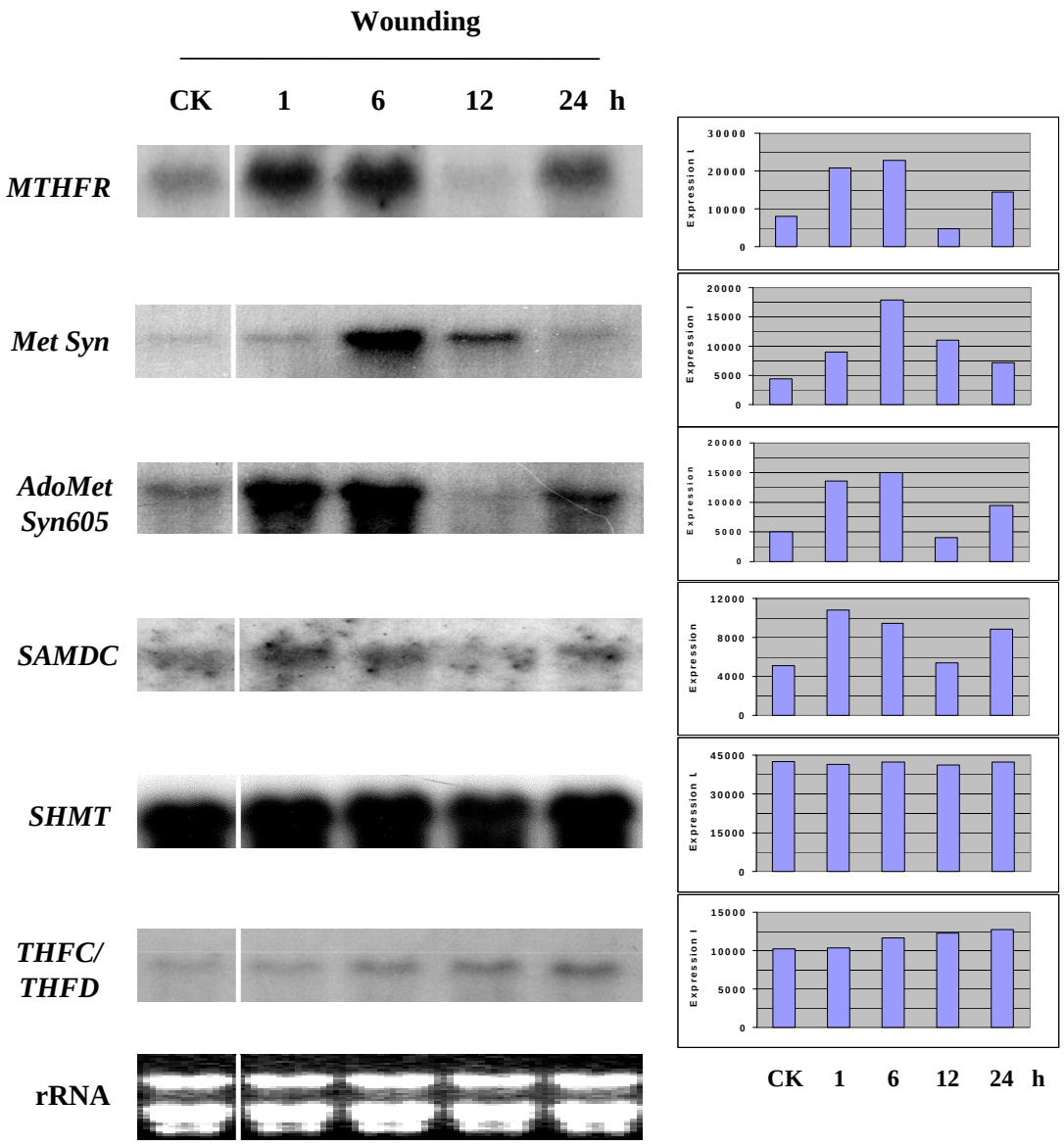

Fig. 4.22 Northern blot analysis of *T. monococcum MTHFR, Met Syn, AdoMet Syn605, SAMDC, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to wounding. Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager^{TM} 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

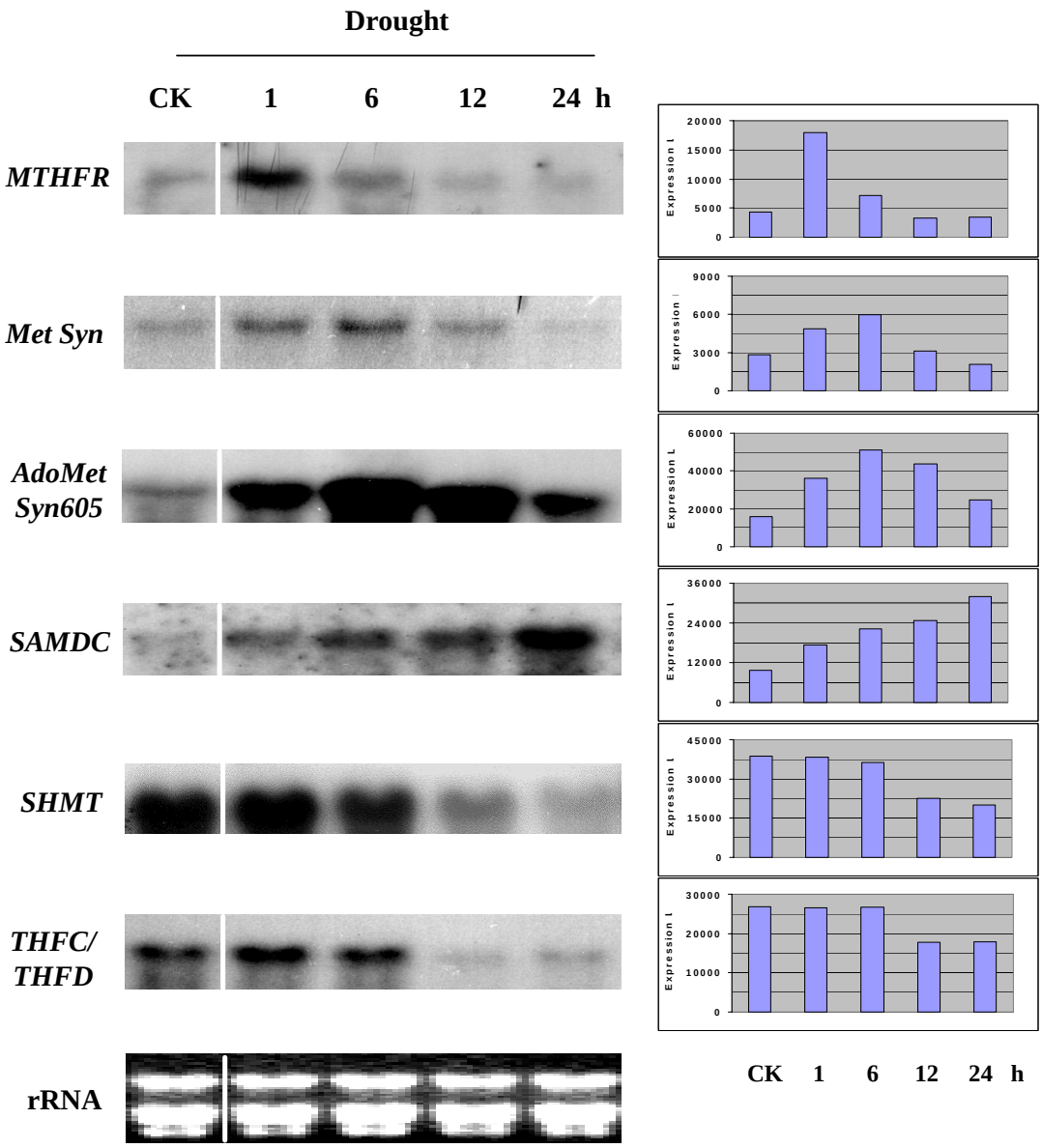

Fig. 4.23 Northern blot analysis of *T. monococcum MTHFR, Met Syn, AdoMet Syn605, SAMDC, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to drought. Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager^{TM} 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.4.3 Cold

To examine whether the genes involved in the generation and supply of methyl units are involved in defense in response to cold stress, total RNA was extracted from 10-day-old Tm441 plants after treatment with cold. Three genes *MTHFR, Met Syn and AdoMet605* showed the same response after treatment with cold stress: Transcription of all three was weakly induced at 1 h and increased to the maximum level at 24 h, after which a decrease occurred (Fig. 4.24).

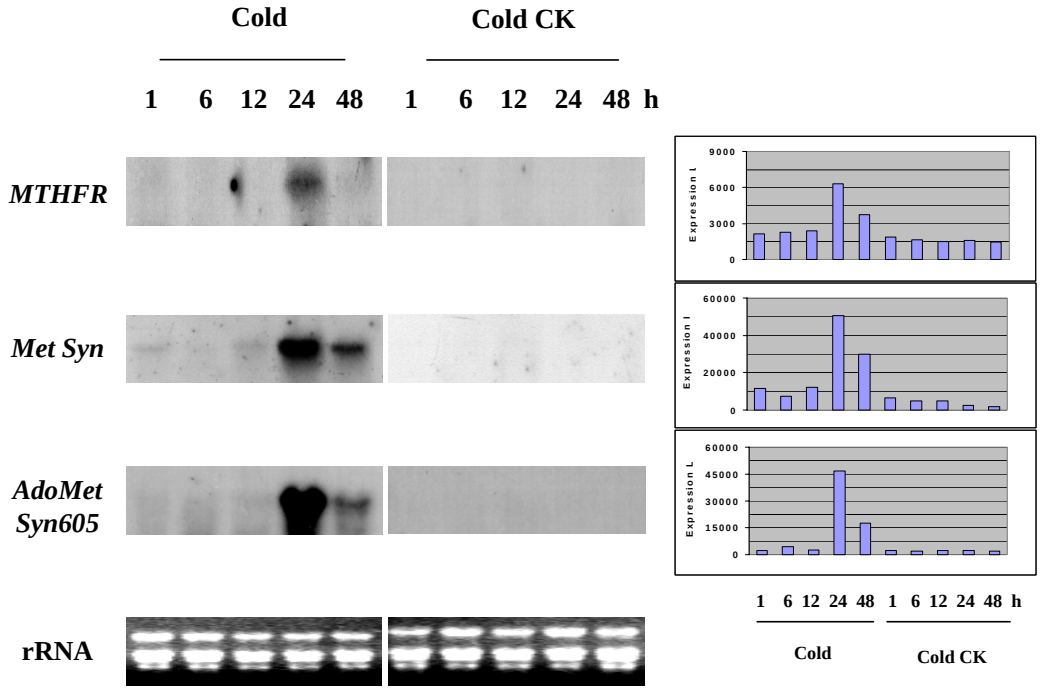

Fig. 4.24 Northern blot analysis of *T. monococcum MTHFR, Met Syn and AdoMet Syn605* expression from leaves of 10-day-old seedlings of Tm441 in response to cold. Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12, 24 and 48 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager™ 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.4.4 Sodium chloride

To examine the effect of NaCl stress on the gene expression, total RNA was extracted from 10-day-old Tm441 plants after treatment with salt. The results indicated that *MTHFR* and *Met Syn* were coordinately expressed. The mRNA level of *MTHFR* was induced at 12 h, increased to the maximum at 24 h and then decreased at 48 h. However, after salt treatment, *SHMT* has the same expression pattern compared to the control level (Fig. 4.25).

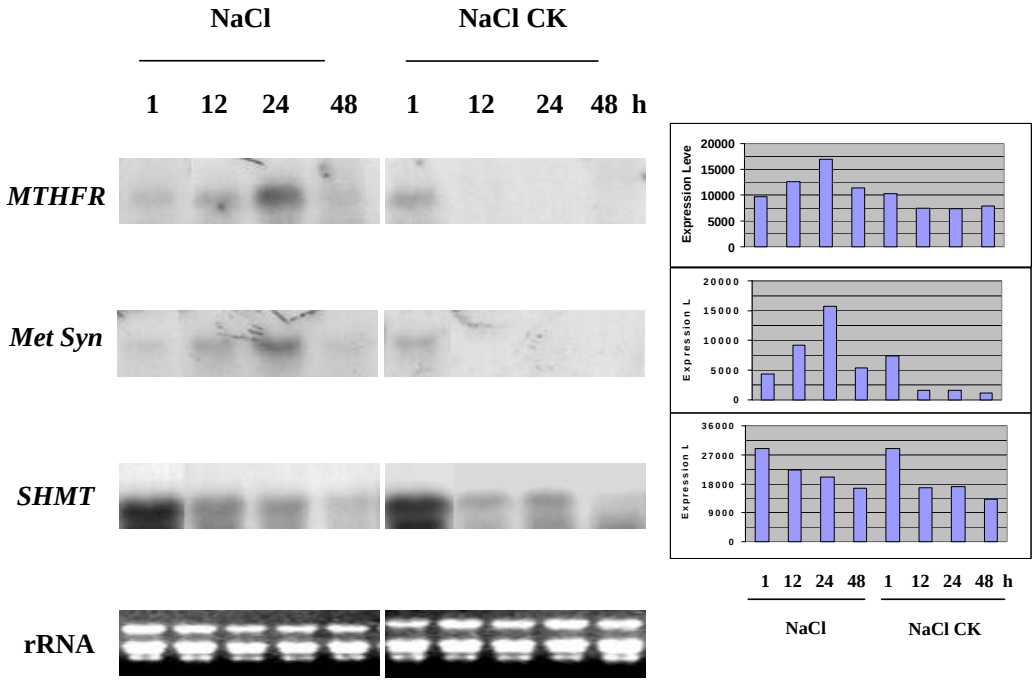

Fig. 4.25 Northern blot analysis of *T. monococcum MTHFR, Met* Syn and *SHMT* expression from leaves of 10-day-old seedlings of Tm441 in response to NaCl. Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 12, 24 and 48 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager^{TM} 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.5 Expression of genes in the generation and supply of methyl units in response to treatments with stress signaling molecules

Stress signaling molecules play an important role in plant defense. To study the detail of a series of genes involved in the generation and supply of methyl units in response to stress signaling compounds, time course experiments were conducted after treating leaves of Tm441 with H_2O_2 (10 mM), ethephon (releasing ethylene) (100 μM), MeJA (200 μM), SA (5 mM), ABA (100 μM) and sodium nitroferricyanide (III) dihydrate (releasing NO) (10 mM).

4.5.1 H_2O_2 and ethephon

Expression patterns for group I genes *MTHFR, Met Syn, AdoMet2661* and *AdoMet605* were similar. The induced expression occurred within 1 h for H_2O_2 treatment and in 6 h for ethephon treatment. The transcription levels of the entire group I genes reached a peak either in 6 h or 12 h and then decreased thereafter (Fig. 4.26).

The group II genes, *SHMT and THFC/THFD,* were almost constitutively expressed throughout the time course in response to H_2O_2 and ethephon (Fig. 4.26).

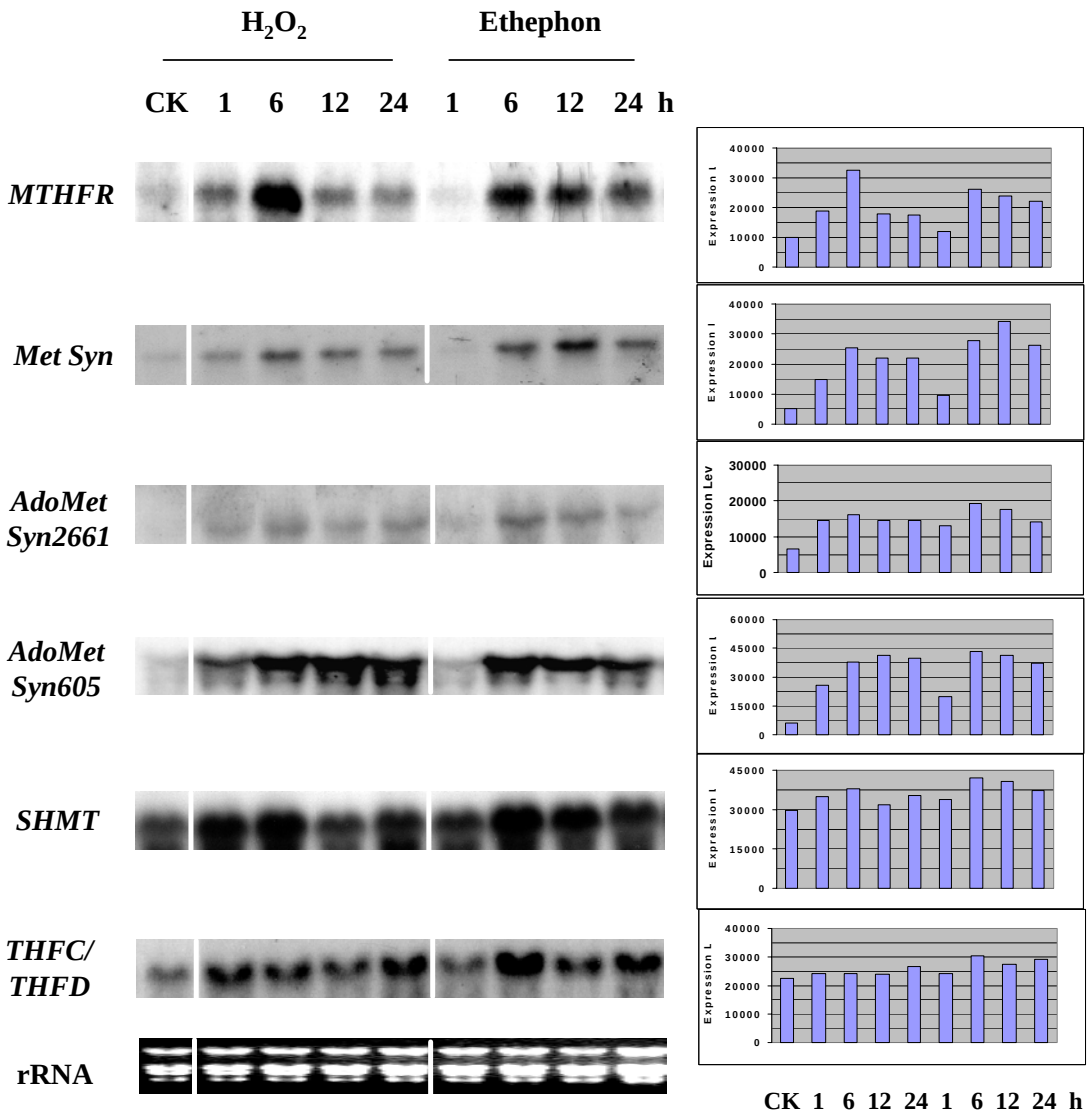

Fig. 4.26 Northern blot analysis of *T. monococcum MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to H_2O_2 (10 mM) and ethephon (100 µM, releasing ethylene). Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager™ 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.5.2 MeJA and SA

RNA from leaves of 10-day-old seedlings of Tm441were harvested after spraying with MeJA (200 μM) or SA (5 mM). mRNA levels were then followed for a further 24 h by northern analysis.

In response to MeJA and SA, *Met Syn* transcription was first detected at 1 h, was increased further at 6 h and reached a maximum at 12 h before decreasing. *AdoMet605* mRNA accumulation was essentially the same as that of *Met Syn* (Fig. 4.27).

Transcripts of *SHMT and THFC/THFD* were constitutively expressed from 1 h to 24 h when compared to control levels (Fig. 4.27).

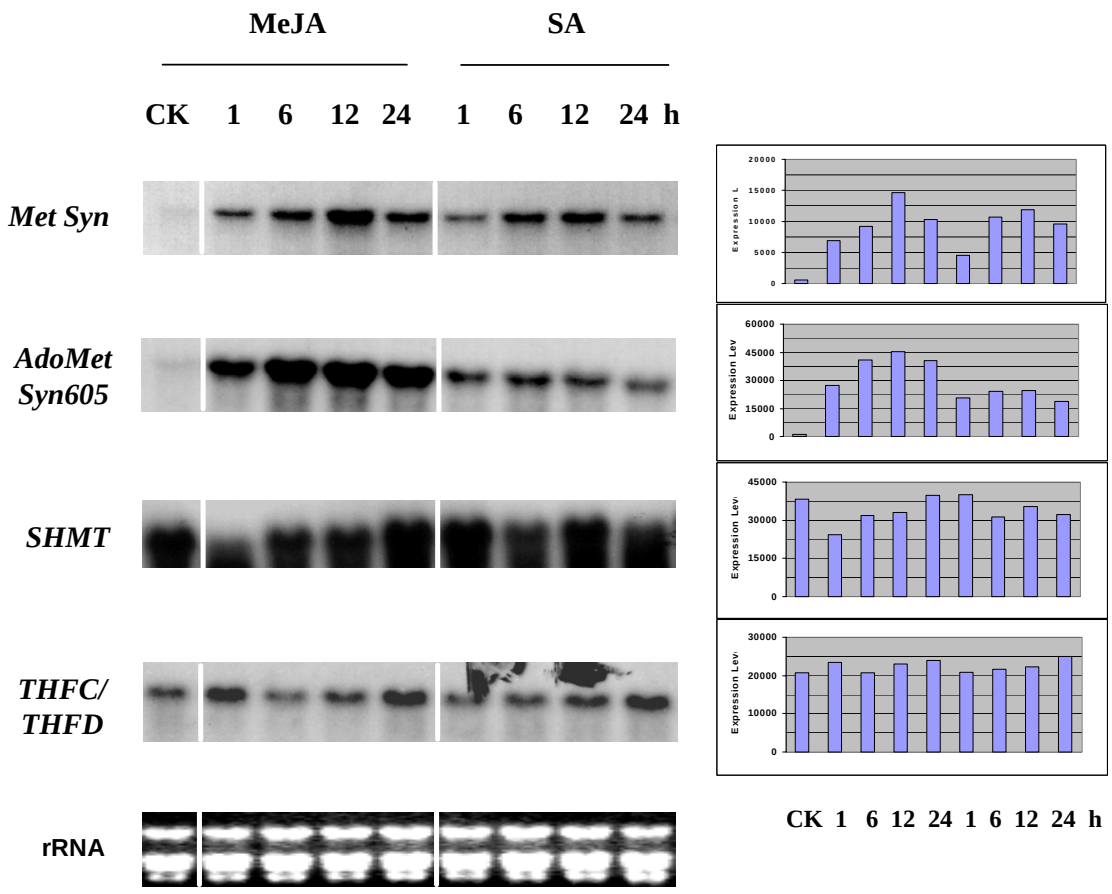

Fig. 4.27 Northern blot analysis of *T. monococcum Met Syn, AdoMet Syn605, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to MeJA (200 µM) and SA (5 mM). Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager^{TM} 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

4.5.3 ABA

mRNA levels for *Met Syn*, *AdoMet605* and *SAMDC* were coordinately expressed after ABA (100 μM) treatment. The transcript level was initially induced at 1 h, decreased at 6 h and then increased to the maximum level at 12 h to 24 h (Fig. 4.28). Transcripts of *SHMT and THFC/THFD* were coordinately expressed. Expression of both genes was down-regulated at 6 h and then increased until 24 h (Fig. 4.28).

4.5.4 NO

NO was found to function as a stress signal molecule recently (Durner and Klessig, 1999). Sodium nitroferricyanide (III) dihydrate (10 mM), which releases NO, was sprayed on 10-day-old wheat plants and RNA extracted at the times indicated (Fig. 4.29). *MTHFR, Met Syn* and *AdoMet605* have similar induction patterns. The transcript of the 3 genes was induced within 1 h, increased to the highest level at 12 h and declined thereafter. Expression of group II genes *SHMT and THFC/THFD* was down-regulated at 6 h and 12 h and then increased at 24 h (Fig. 4.29).

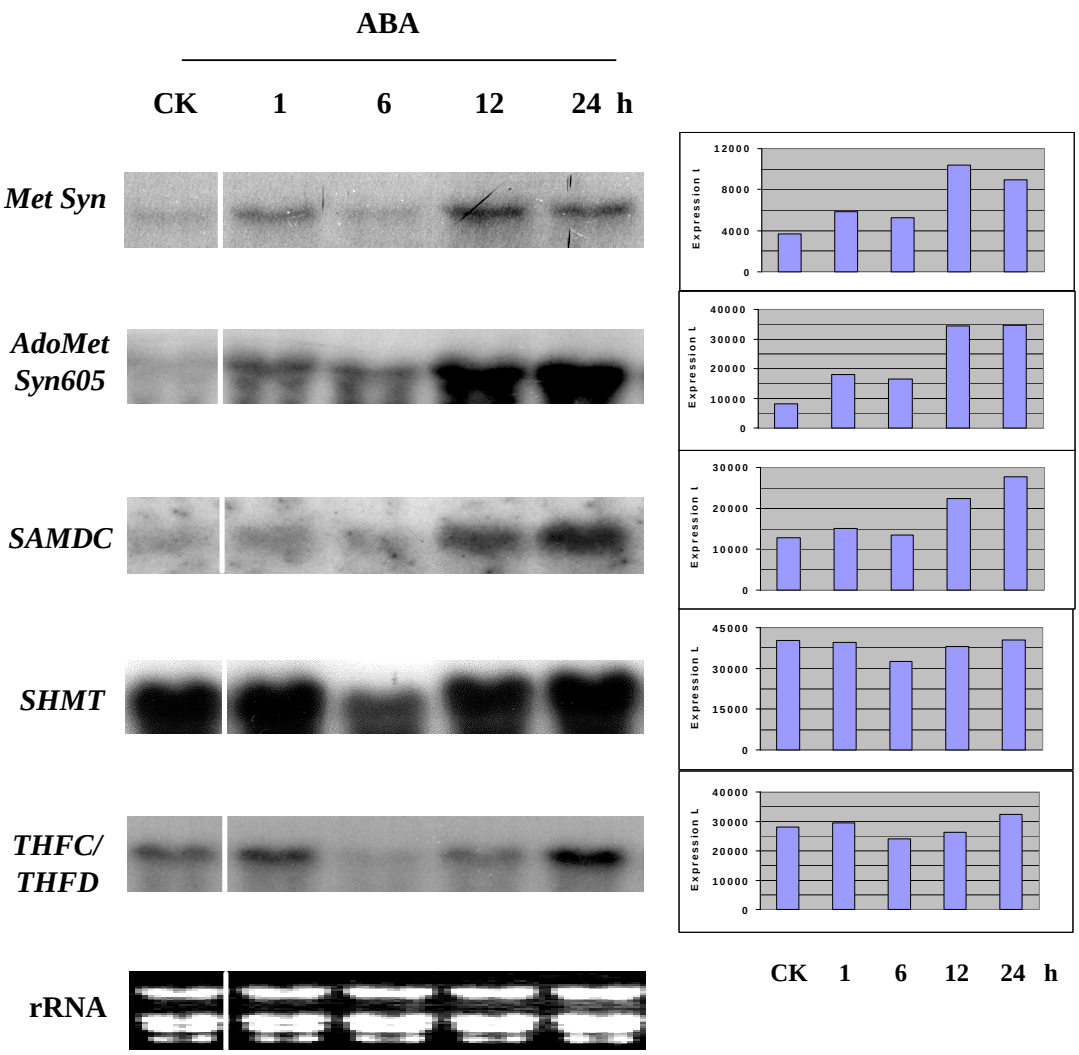

Fig. 4.28 Northern blot analysis of *T. monococcum Met Syn, AdoMet Syn605, SAMDC, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to ABA (100 µM). Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded in each lane on a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager™ 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

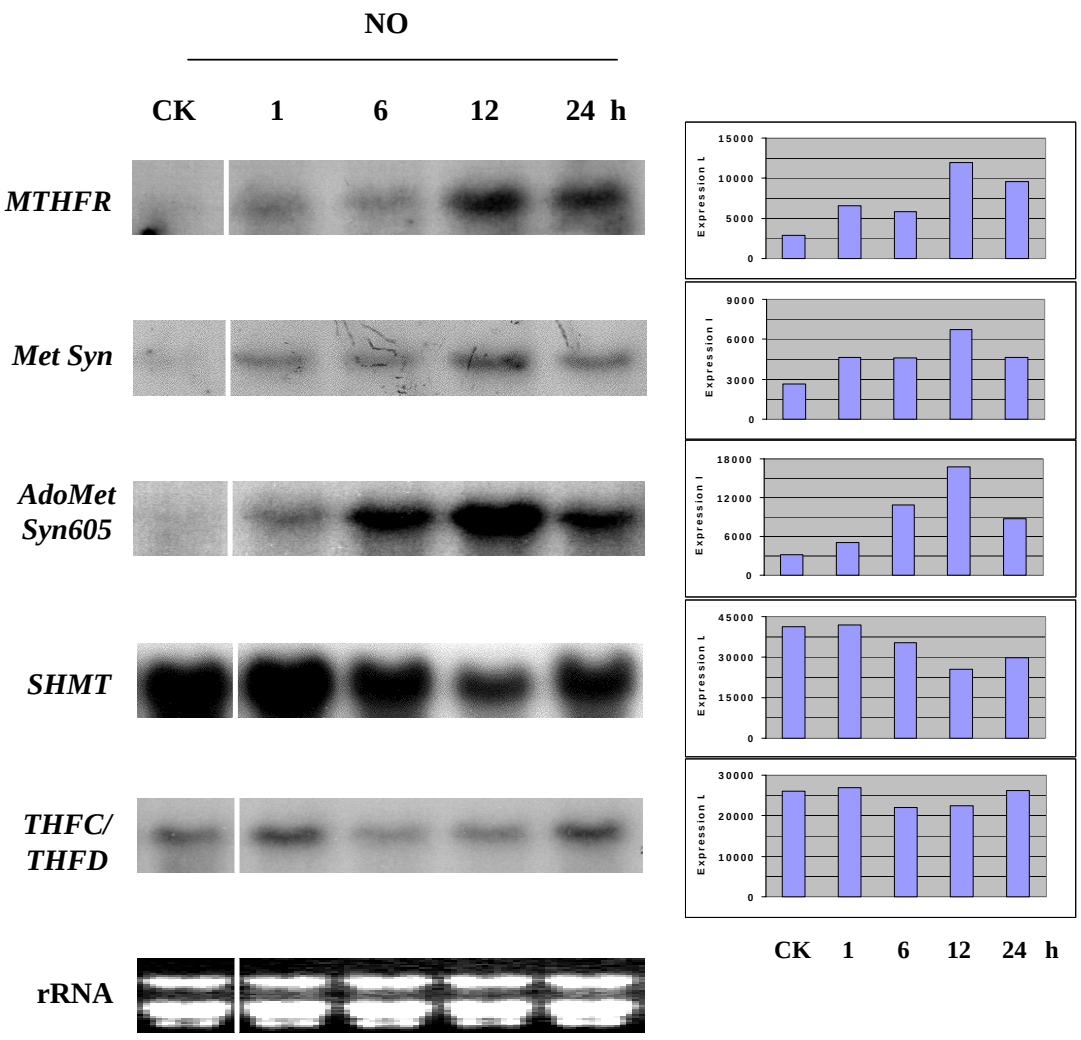

Fig. 4.29 Northern blot analysis of *T. monococcum MTHFR, Met Syn, AdoMet Syn605, SHMT* and *THFC/THFD* expression from leaves of 10-day-old seedlings of Tm441 in response to NO (10 mM sodium nitroferricyanide (III) dihydrate releasing NO). Total RNA was isolated from non-treated Tm441 leaves (CK) and treated leaves at 1, 6, 12 and 24 h as described in the text. A 20 µg aliquot of total RNA was loaded on each lane in a 1.2% gel, blotted to a Zetaprobe membrane and hybridized with ^{32}P-labeled cDNAs. Even loading of the RNA was confirmed by using a ribosomal RNA control. The densitometry of the transcripts was performed by Alphalmager™ 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA, USA).

5. DISCUSSION

5.1 Group I genes in response to biotic and abiotic stresses

5.1.1 Group I genes in response to biotic stress

To help answer the questions posed in the introduction, a cDNA library was used that was constructed by others from leaf epidermis of diploid wheat (*Triticum monococcum*) 24 h after inoculation with powdery mildew fungus *Bgt.* Expressed sequence tag analysis revealed a group of genes, including *MTHFR*, *Met Syn*, *AdoMet Syn2661*, *AdoMet Syn605*, *SAMDC*, *SHMT* and *THFC/THFD,* which are involved in C-1 metabolism. To investigate further the mRNA accumulation of C-1 related genes in response to biotic stress, 10-day-old compatible and incompatible *T. monococcum* plants were inoculated with *Bgt* and total RNA was extracted from these plants during a 144 hpi time course.

Northern results showed that in the compatible host interaction*, Tm-MTHFR, Tm-Met Syn, Tm-AdoMet Syn2661, Tm-AdoMet Syn605 and Tm-SAMDC* are coordinately induced by powdery mildew infection and that all 5 genes have two expression peaks at 6 and 24 hpi (Fig. 4.20). Peaks at 6 hpi are consistent with fungal primary germ tube penetration of the plant surface; peaks at 24 hpi are correlated with appressorial germ tube penetration.

In the incompatible interaction, the transcripts of the 5 genes are coordinately regulated but the expression pattern is different from that of the compatible interaction.

In the resistant line Tm453, transcripts of the 5 genes reach a maximum at 24 hpi (Fig. 4.21), which follows the time of formation of a large papilla underneath the appressorial germ tube penetration peg; this can block further penetration of the pathogen. The induction of the transcription level of these 5 genes in both compatible and incompatible interactions implies that they play a defense role in response to biotic stress.

It has been reported that in parsley and alfalfa cells, *AdoMet Syn* gene expression was induced in response to fungal elicitor and yeast cell wall, respectively (Kawalleck et al., 1992; Gowri et al., 1991), suggesting a role for *AdoMet Syn* in plant defense against pathogen attack. AdoMet acts as a methyl group donor in numerous highly specific transmethylations, such as proteins, nucleic acids, polysaccharides and phenylpropanoid derivatives (Tabor and Tabor, 1984). Moreover, AdoMet also serves as a propylamine group donor in the biosynthesis of polyamines and has an important role as an intermediate in ethylene biosynthesis (Van Doorsselaere et al., 1993; Yang and Hoffman, 1984). Because high levels of AdoMet synthesis and the turnover of the activated methyl cycle are required for these reactions to proceed at elevated rates, the basic level of gene expression may not suffice under stress conditions, thus resulting in an increased mRNA expression level of the genes involved in these pathways.

Expression of *Tm-AdoMet Syn2661* and *Tm-AdoMet Syn605* genes is coordinately induced by powdery mildew infection in both compatible and incompatible host interactions (Fig. 4.20; Fig. 4.21). The *Tm-AdoMet Syn2661* and *Tm-AdoMet Syn605* expression peaks at 6 and 24 hpi are consistent with the formation of primary and appressorial germ tubes, both of which induce the formation of host cell wall appositions (Thordal-Christensen et al., 1997). Comparison of the *Tm-AdoMet Syn2661*

and *Tm-AdoMet Syn605* sequences shows that the coding regions are highly conserved: 88.2% of the nucleotide sequences and 96.8% of the deduced amino acid sequences are identical, which is similar to the equivalent genes in *Arabidopsis, sam1* and *sam2* (88.7% and 96.7% respectively, Peleman et al., 1989b). Peleman et al. (1989b), using 3' untranslated region (UTR) gene specific probes in hybridization experiments, showed that *Arabidopsis sam1* and *sam2* had similar expression patterns in different organs, which might be explained by the three highly conserved sequences in the promoter region of the two *sam* genes. Neither *Tm-AdoMet Syn2661* nor *Tm-AdoMet Syn605* contained extended promoter sequences. Therefore, we do not know whether or not the similar expression patterns were attributable to the same conserved sequences in the promoter region.

Northern blot signals indicate that *Tm-AdoMet Syn605* has more abundant transcript than that of *Tm-AdoMet Syn2661*, thus probably accounting for the majority of AdoMet Syn activity. Based on the degree of amino acid sequence similarity, AdoMet Syns have been grouped into two different types I and II, suggesting differences in the properties or the regulation for two distinct enzymes (Schröder et al., 1997). *Arabidopsis sam1* and *sam2,* as well as *Tm-AdoMet Syn2661* and *Tm-AdoMet Syn605* all belong to type I. In my experiments, only the type I gene was found in wheat epidermal EST collection; to identify a type II gene and study whether the expression patterns of these two types of genes are differentially regulated in response to stresses in wheat would be valuable.

5.1.2 Group I genes in response to abiotic stresses

In my study, *Tm-MTHFR*, *Tm-Met Syn*, *Tm-AdoMet Syn605* and *Tm-SAMDC* gene expression was also up-regulated in response to abiotic stresses, such as wounding and

drought. As we know, wounding can increase expression of most plant defense related genes, so investigating the response of the genes to wounding would be useful.

The expression pattern of *Tm-MTHFR*, *Tm-AdoMet Syn605* and *Tm-SAMDC* has two peaks, at 6 and 24 hpi after wounding treatment (Fig. 4.22). This may imply similar mechanisms of pathogen and wounding defense. Lignification is thought to be the plant response to wounding and mechanical stresses (Boudet, 2000). Moerschbacher et al. (1990) demonstrated that chemical inhibition of lignification in wheat resulted in a decreased resistance to *Puccinia graminis.* Cell wall can resist infection by a pathogen through the deposition of lignin. Lignin monomers need to be methylated before polymerization resulting in the consumption of increased amounts of AdoMet. Thus, the up-regulation of the genes encoding enzymes in the pathway of generation and supply of methyl units after wounding treatment might be explained by a demand for more AdoMet as methyl group donor for lignin methylation. Other research has shown that *AdoMet Syn* gene expression has a relationship with lignin biosynthesis. For example, in *Arabidopsis*, the *AdoMet Syn* gene is expressed primarily in the vascular tissue where a great deal of lignin biosynthesis occurs (Peleman et al., 1989a). However, northern blot analysis of rice indicated a similar *AdoMet Syn* mRNA levels in root, leaf and cell suspensions (Van Breusegem et al., 1994), which differs from *Arabidopsis* where expression was high in root and stem compared to leaves (Peleman et al., 1989a). This might be explained by the high percentage of lignified tissue in rice leaves compared to dicot plants.

Van Breusegem et al. (1994) identified a correlation between rice *AdoMet Syn* expression and dehydration stress resulting in a 2- to 4- fold increased expression of

AdoMet Syn, suggesting that under drought conditions plants need increased amounts of methionine derivatives. My result showed that *Tm-AdoMet Syn605* transcription level was increased in response to drought, which confirmed Van Breusegem's observation. I hypothesized that the synthesis of methionine would also be up-regulated by dehydration stress. My results indicated that not only was *Tm-Met Syn* gene expression up-regulated by drought stress but that the transcription levels of *Tm-SAMDC* and *Tm-MTHFR*, which are downstream and upstream of methionine, respectively, were also increased by drought stress (Fig. 4.23).

Interestingly, the expression of these four genes was up-regulated sequentially: *Tm-MTHFR* had an expression peak at 1 h, *Tm-Met Syn* at 6 h, *Tm-AdoMet Syn605* reached the highest level from 6 - 12 h and *Tm-SAMDC* increased to the maximal level at 24 h (Fig. 4.23). This sequence is correlated with the direction of C-1 flux: Firstly, MTHFR catalyzes the reduction of N^5, N^{10}-methylene-THF to N^5-methyl-THF, which serves as a methyl donor for the formation of methionine from homocysteine by Met Syn. Secondly, AdoMet Syn catalyzes the formation from methionine of AdoMet, which serves as a methyl group donor in highly specific transmethylation reactions (Van Doorsselaere et al., 1993). Finally, SAMDC catalyzes the production of decarboxylated AdoMet, which is the methyl group donor for most cellular methylation reactions and the aminopropyl moiety donor for spermidine and spermine biosynthesis (Walden et al., 1997). In drought stress, it may be that the induced expression of genes involved in the pathways of generation and supply of methyl units is not synchronic but, rather, one gene expression induces the next, downstream, in a chain-reaction.

Recent studies have indicated that *AdoMet Syn* genes are expressed in both an organ-specific and gene-specific manner in response to NaCl stress in *Catharanthus roseus* (Schröder et al., 1997), tomato (Espartero et al., 1994) and rice (Lee et al., 1997). Increased content of methionine enhanced by the resistance of yeast to NaCl and LiCl was also reported by Glaser et al. (1993). Rice *Met Syn* transcription level was increased significantly at 12 h and 24 h and reduced sharply at 48 h after exposure to alkaline and NaCl stresses, suggesting that this gene is involved in adaptation to these stresses (Xie et al., 2002). Similarly, my experiments showed that the transcript level of *Tm-MTHFR* and *Tm-Met Syn* increased with the application of NaCl stress (Fig. 4.25). Also, the accumulation of *Tm-MTHFR*, *Tm-Met Syn* and *Tm-AdoMet Syn605* mRNAs in response to low temperature stress was tested by RNA hybridization. Results demonstrated that these genes are coordinately expressed (Fig. 4.24). These sets of data, together, suggest an important defense role for the genes involved in the pathways of generation and supply of methyl units under these environmental stresses (Figs. 4.24, 4.25).

5.1.3 Group I genes in response to stress signal molecules

My experimental results showed that the expression of genes involved in the pathway of generation and supply of methyl units was up-regulated in response to biotic and abiotic stresses (Fig. 4.20 - Fig. 4.25). Comprehensive studies of PR genes induced by a variety of pathogens as well as various abiotic stresses and stress signal molecules have been well-established (Van Loon and Van Strien, 1999; Schweizer et al., 1997; Curtis et al., 1997; Grillo et al., 1995; Snowden et al., 1995; Bowling et al., 1994; Brederode et al., 1991; Van Loon, 1985). These results, together, led me to investigate whether the expression of genes encoding enzymes in these pathways was also up-regulated by some

stress signal molecules. To the present, there is very limited information about the response of these genes to such molecules. To my knowledge, only the effect of ABA treatment on tomato *AdoMet Syn*, wheat and rice *SAMDC*, and MeJA on barley and tobacco *SAMDC* has been reported (Espartero et al., 1994; Li and Chen, 2000a, 2000b; Biondi et al., 2001; Walters et al., 2002). Therefore, investigating gene expression in the pathways of generation and supply of methyl units in wheat plants treated with a range of stress signaling molecules would be expected to yield new information.

It is now clear that H_2O_2 is a signaling molecule in plants (Foyer et al., 1997; Dat et al., 2000; Neil et al., 2002). H_2O_2 generation is induced in plants following a series of biotic and abiotic stresses, including UV irradiation, low temperature, dehydration, wounding, ABA and pathogen challenge (A-H-Mackerness et al., 2001; Prasad et al., 1994; Guan and Scandalios, 2000; Pei et al., 2000; Lamb and Dixon, 1997). H_2O_2, acting as a signal, can induce a variety of molecular and physiological responses in plants. It has been established that H_2O_2 generation in response to biotic stress drives the cross-linking of cell wall proteins (Bradley et al., 1992) and phenolics (Grant and Loake, 2000). H_2O_2 also modulates gene expression during defense responses. Thus, H_2O_2 induced the expression of *PAL* in *Arabidopsis* and the expression of the defense-related genes for glutathione *S*-transferase (*GST*) in both soybean and *Arabidopsis* (Desikan et al., 1998; Levine et al., 1994). In my experiment, the genes, *Tm-MTHFR, Tm-Met Syn, Tm-AdoMet2661* and *Tm-AdoMet605,* were all up-regulated in response to H_2O_2 (Fig. 4.26) suggesting that H_2O_2 may be a factor, directly or indirectly, in the signal transduction pathway that regulates expression of these genes.

I also showed that *Tm-MTHFR*, *Tm-Met Syn*, *Tm-AdoMet Syn2661* and *Tm-AdoMet605* are coordinately expressed by ethephon treatment, a compound that releases ethylene (Fig. 4.26). Increased ethylene biosynthesis is a general response of plants to many environmental stresses (Liu and Zhang, 2004; Bleecker and Kende, 2000; Yip and Yang, 1998). An accelerated synthesis of ethylene might create a demand for a greater supply of AdoMet, so it may induce the expression of *AdoMet Syn* and other related genes.

Both MeJA and SA have been proposed as key regulators of plant response to wounding and pathogens (Blechert et al., 1995; Ryan, 1990). The transcripts of *Tm-Met Syn* and *Tm-AdoMet Syn605* were coordinately induced by MeJA and SA treatments (Fig. 4.27), suggesting that both MeJA and SA are involved, directly or indirectly, in regulating expression of these two genes.

ABA is known to be implicated in responses of plants to various environmental stresses, and most stress-inducible genes are induced by exogenous ABA treatment (Chinnusamy et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 1996; Grill and Himmelbach, 1998). My results showed that *Tm-Met Syn*, *Tm-AdoMet Syn605* and *Tm-SAMDC* are affected by ABA treatment (Fig. 4.28), suggesting activation of the genes through ABA-dependent or -related pathways. However, reduction in the level of the transcripts of the genes 6 h after ABA treatment was observed (Fig. 4.28). It has been reported that the rice *SAMDC1* gene was detected at 3 h, decreased at 12 h and increased to the maximum level at 48 h in response to ABA treatment (Li and Chen, 2000b), which is a similar expression pattern to that of my *Tm-SAMDC*. The negative effects of

ABA on transcript accumulation probably occur at the post-transcriptional level by affecting mRNA stability and turnover (Li and Chen, 2000b).

Recent studies have demonstrated that nitric oxide (NO) is a signal molecule in plants (Bolwell, 1999; Durner and Klessig, 1999). The rapid induction of NO synthesis by bacterial challenge in soybean and *Arabidopsis* suspension cultures was reported recently (Delledonne et al., 1998; Clarke et al., 2000). NO also activates the expression of the defense-related genes *PAL1, PR-1* and *GST* during plant pathogen interactions (Delledonne et al., 1998; Durner et al., 1998). NO generation has been detected under conditions in which H_2O_2 generation is also stimulated (Delledonne et al., 1998; Clarke et al., 2000). My finding that expression of *Tm-MTHFR, Tm-Met Syn, Tm-AdoMet Syn605* is all up-regulated by NO treatment (Fig. 4.29) suggest that NO acts as a signal in defense responses, directly or indirectly.

My results demonstrate the induction of genes in the pathways of generation and supply of methyl units in response to all treatments, including biotic and abiotic stresses and stress signal molecules, suggesting that these pathways are components of a general response to stresses.

5.2 Group II genes in response to biotic and abiotic stresses

Serine is a key intermediate in a number of important metabolic pathways and is also the principal C-1 donor for synthesis of nucleic acids, proteins and in the interconversion of homocysteine and methionine (Walton and Woolhouse, 1986; Appling, 1991; Ireland and Hiltz, 1995). Serine can be formed by several pathways, the principal one being the glycolate pathway, associated with the GDC and photorespiratory glycine metabolism through the THF-dependent conversion of glycine

to serine by SHMT (Prabhu et al., 1996; King, 2002). An alternative THF-dependent pathway of serine biosynthesis can occur via the C1-THF synthase (THFC/THFD and N^{10}-formyl-THF synthetase) and SHMT using formate as the C-1 source (Prabhu et al., 1996; King, 2002). A third is the phosphorylated pathway which includes three enzymes: PGDH, PSAT and PSP. A fourth is the non-phosphorylated pathway, which involves four enzymes: PGP, GDH and one or two aminotransferases, AHAT and GHAT.

Notably, no *GDC* has been found in the 2700 EST collection used in these studies. Taylor et al. (2002) revealed that environmental stress causes oxidative damage to plant mitochondria, which can inhibit GDC activity. The cDNA library I used was constructed from mRNA of susceptible wheat (*Triticum monococcum*) line 441 epidermis, 24 h after inoculation with powdery mildew fungus (*Bgt*), which may explain the absence of the *GDC* transcript.

Previous reports from this laboratory have demonstrated that the two THF-linked pathways of serine biosynthesis operated independently of one another in *Arabidopsis* and that the flux through the GDC/SHMT pathway is the major source of C-1 (Prabhu et al., 1996; King, 2002). My studies showed that expression of *Tm-SHMT* and *Tm-THFC/THFD* was either constitutive or down-regulated in all treatments (Fig. 4.20 - 4.29), which is different from *Tm-MTHFR*, *Tm-Met Syn*, *Tm-AdoMet Sy2661*, *Tm-AdoMeet Syn605* and *Tm-SAMDC*. These observations might be explained by the fact that both *SHMT* and *THFC/THFD* are involved in pathways crucial to many central activities in cells, such as serine biosynthesis for the generation of C-1 units,

thymidylate and purine biosynthesis in general, and are, therefore, not linked specifically only to responses to stresses.

Plants grown at increased salinity and low temperature are stimulated to accumulate serine, suggesting that this amino acid may have significance in response to various stresses (Stewart and Larher, 1980; Draper, 1972). Stresses such as flooding, low temperature and high salinity also can increase the transcription level of the enzymes involved in the phosphorylated serine biosynthetic pathway, but not in the glycolate pathway (Ho and Saito, 2001). These results demonstrated that the phosphorylated pathway might play a key role in response to environmental stresses.

5.3 Sequence analysis and prediction for subcellular localization

5.3.1 MTHFR

Tm-MTHFR proteins have an N-terminal catalytic domain homologous to the *E. coli* enzyme and also have an approximately 270-residue C-terminal (Fig. 4.2). When compared to plant, mammalian, yeast and bacterial counterparts, Tm-MTHFR appears to be a cytosolic protein because it lacks obvious targeting sequences (*e.g.* mitochondrial or chloroplast transit peptides). Roje et al. (1999) found that *Arabidopsis* and maize MTHFR enzymes strongly prefer NADH to NADPH and are not inhibited by AdoMet, which is different from mammalian MTHFR. The lack of inhibition of plant MTHFRs by AdoMet seems attributable to the absence of an AdoMet binding site. The human enzyme contains a putative AdoMet binding site in the C-terminal domain (Fig. 4.2). Plant MTHFRs use NADH rather than NADPH and the ratio of NADH/NAD is 10^{-3}, suggesting that the plant MTHFR reaction is reversible (Roje et al., 1999), which may explain the absence of inhibition by AdoMet of the plant enzymes. The reversible

reaction can keep an adequate pool of N^5, N^{10}-methylene-THF for thymidylate and purine synthesis, which does not need a feedback signal from AdoMet. Up to the present, there is very little information about MTHFR, which is the least understood enzyme of folate-mediated C-1 metabolism in plants. To my knowledge, there is no report on this gene in response to biotic and abiotic stresses. Isolation and characterization of *Tm-MTHFR* would greatly help investigations into THF-mediated C-1 metabolism in response to stresses in plants.

5.3.2 Met Syn

Sequence analysis indicated that a C-terminal motif is present in this enzyme in plants and microorganisms (Fig. 4.5). It seems that this motif is strictly conserved in cobalamin-independent Met Syns. The highly reactive Cys726 (Fig. 4.5) embedded in this 11-amino-acid motif found in *E. coli* possibly functioned as an intermediate methyl acceptor in catalysis, which is analogous to the role of cobalamin in the reaction catalyzed by the cobalamin-dependent enzyme (González et al., 1992). The cysteine residue in this motif functioned as a zinc ligand indicating that this metal is necessary for catalysis (González et al., 1996).

The localization of Met Syn protein is still a matter of controversy. Enzyme activity has been assigned to the cytosol but has been found also in chloroplasts and mitochondria (Shah and Cossins, 1970; Clandinin and Cossins, 1974). However, most data have demonstrated that the protein is localized in the cytosol, for instance in the case of *Catharanthus roseus* (Eichel et al., 1995), *Chlamydomonas reinhardtii* (Kurvari et al., 1995), *Arabidopsis thaliana* (Gakière et al., 1999) and *Solanum tuberosum* (Zeh et al., 2002). In the present studies, only a partial cDNA of Tm-Met Syn was isolated; no

signal peptide or hydrophobic stretch information could be obtained from the N-terminal end. Furthermore, the phylogenetic tree demonstrated that Tm-Met Syn from this wheat was in the cytosolic, rather than chloroplastic, Met Syn group, based on amino acid similarity (Fig. 4.6).

5.3.3 AdoMet Syn

Based on the degree of amino acid sequence similarity, AdoMet Syns have been put into two groups, type I and type II (Schröder et al., 1997). *Catharanthus roseus, Actinidia chinensis* and *Lycopersicon esculentum* have both types (Schröder et al., 1997; Whittaker et al., 1995; Espartero et al., 1994). From the phylogenetic tree relationship, both Tm-AdoMet Syn2661 and Tm-AdoMet Syn605 belong to type I (Fig. 4.10). The amino acid sequence contains two conserved AdoMet Syns motifs, which are present in all plant AdoMet Syns. The first of these is a hexapeptide, which is thought to be involved in ATP binding (Pajares et al., 1991). The second is a glycine-rich nonapeptide possibly involved in binding the triphosphate of ATP (Takusagawa et al., 1996) (Fig. 4.9).

Hydrophobic analysis of the amino acid sequence showed the lack of a transit peptide region at the N-terminus of the Tm-AdoMet Syn, suggesting that *Tm-AdoMet Syn* encodes a cytosolic enzyme, as in the case of other AdoMet Syns.

5.3.4 SAMDC

The 5'-untranslated leader sequences of SAMDC, which were 469 bp, 472 bp, and 502 bp long for *Catharanthus roseus*, carnation CSDC9 and carnation CSDC16, respectively, have been found to include small uORFs encoding a highly conserved

peptide of about 50-54 residues (Schröder and Schröder, 1995; Lee et al., 1997). Results from the present experiments showed that Tm-SAMDC contained an unusually long 5'-untranslated region of 517 nucleotides that encoded a putative 49 amino acid polypeptide sequence (Fig. 4.11).

In mammals, the 5'-untranslated leader is about 330 nucleotides long (Franceschetti et al., 2001). The Tm-SAMDC sequence contains a 49 amino acid upstream open reading frame (uORF) in its 5'-untranslated region, which was conserved in the 5' leaders of other plant SAMDCs mRNAs. No similarity was found between the long leader sequences of plant and the SAMDCs in other organisms. Some papers have reported that SAMDC uORF acts as a negative regulatory element that decreases the translational efficiency of SAMDC mRNA in animals (Hill and Morris, 1992; Shantz et al., 1994). As there is no similarity between plants and animals, the uORF in plants may have evolved a different type of translational regulation.

The second serine in the conserved sequence, LSESS (Fig. 4.12), which was identified as a processing site for the proenzyme, has been found in yeast, humans and plants (Kashiwagi et al., 1990; Stanley et al., 1989; Lee et al., 1997; Xiong et al., 1997). The cleavage of SAMDC proenzyme, resulting in the formation of a small β-chain in the N-terminus and a larger α-chain in the C-terminus, has been reported and was confirmed to be essential for enzyme activity (Schröder and Schröder, 1995; Lee et al., 1997). A putative PEST sequence is highly conserved in all organisms (Fig. 4.12). PEST sequences are associated with the degradation of rapidly turning-over proteins (Roger et al., 1986). The fact that the conserved sequence is found in all organisms suggests structural and functional similarities among the SAMDC enzymes in all eukaryotes.

The subcellular localization predicted by TargetP, together with phylogenetic tree analysis, showed that the predicted amino acid sequence of Tm-SAMDC contained no transit peptide for chloroplastic or mitochondrial localization, suggesting that *Tm-SAMDC* encodes a cytosolic SAMDC (Fig. 4.13).

5.3.5 SHMT

Alignment of the deduced amino acid sequence with previously published SHMT sequences demonstrated considerable conservation along most of the length of the clone. The wheat sequence is more similar to the plant sequences than it is to the human sequence (Fig. 4.15). Interestingly, the wheat sequence is about equally similar to both the human mitochondrial (59% identity) and cytosolic sequences (57% identity). A similar result was found in the case of mitochondrial pea SHMT, which has 74% identity to the mitochondrial and 72% to the cytosolic rabbit sequence (Turner et al., 1992).

SHMT has been purified from mammalian liver (Shostak and Schirch, 1988; Martini et al., 1987; Martini et al., 1989), *Escherichia coli* (Schirch et al., 1985) and plants (Rao and Rao, 1982; Turner et al., 1992). Both cytosolic and mitochondrial SHMTs have been purified from and characterized in mammalian liver (Schirch and Peterson, 1980; Fujioka, 1969). cDNAs of both the cytosolic and the mitochondrial SHMTs have been cloned from human tissue (Garrow et al., 1993). In plants, two immunologically indistinguishable forms of SHMTs have been purified from pea leaf mitochondria, one of which has been sequenced (Turner et al., 1992). A full-length mitochondrial SHMT has been isolated from potato (Kopriva and Bauwe, 1995). There is limited evidence

showing that at least one form of SHMT is located in plastids (Walton and Woolhouse, 1986).

In my study, a cDNA clone encoding a putative SHMT, designated *Tm-SHMT*, was obtained from the 441E-cDNA library. TargetP showed that the predicted amino acid sequence of Tm-SHMT contained transit peptides for mitochondrial localization; phylogenetic tree analysis also indicated that Tm-SHMT was grouped among mitochondrial SHMTs (Fig. 4.16).

5.3.6 THFC/THFD

A gene encoding THFC/THFD was isolated from *T. monococcum* and was named *Tm-THFC/THFD*. The Tm-THFC/THFD ORF consists of 876 nucleotides, which predicts a protein sequence of 292 amino acids (Fig. 4.17). The predicted molecular weight is 30.8 KDa, which is similar to those of *E. coli* (31 KDa, D'Ari and Rabinowitz et al., 1991) and *S. cerevisiae* (36 KDa, West et al., 1993). A search of the GenBank database indicated a high degree of similarity to the bifunctional THFCs/THFDs (46.5% - 88.7%) and low degree to the monofunctional *S. cerevisiae* THFD (16.8%). This result implied that the Tm-THFC/THFD is a bifunctional enzyme. A highly conserved sequence of 13 amino acids located in the C-terminal section was found in the bifunctional proteins but was absent in the yeast monofunctional enzyme (Fig. 4.18). This might be an important binding site for the substrates involved in the dehydrogenase-cyclohydrolase activities of these proteins.

THF-mediated C-1 metabolism plays an important role in several cellular processes including protein, nucleic acid, and amino acid biosynthesis, as well as vitamin

metabolism. It can be required for de novo thymidylate synthesis by thymidylate synthase; it can also be oxidized to N^{10}-formyl-THF or be reduced to N^{5}-methyl-THF.

Phylogentic tree analysis indicated that Tm-THFC/THFD was grouped with cytosolic, rather than mitochondrial and chloroplastic THFCs/THFDs, based on amino acid similarity (Fig. 4.19). TargetP showed that Tm-THFC/THFD contained no transit peptide for chloroplastic or mitochondrial localization again suggesting that *Tm-THFC/THFD* encodes a cytosolic Tm-THFC/THFD.

6. CONCLUSIONS

This project was directed at an understanding of the connections between C-1 metabolism and biotic and abiotic stresses. Stresses trigger a wide range of plant responses, from alteration of transcription, translation and cellular metabolism to changes in growth rates and crop yields (see Literature Review). In my project, northern blots were used to test the transcription levels of the genes in C-1 metabolism in response to a series of biotic and abiotic stresses.

In this project, I identified a series of cDNA sequences encoding MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605, SAMDC, SHMT and THFC/THFD in the pathways of generation and supply of methyl units. These are from a cDNA library of mRNA from a susceptible wheat line 441 epidermis, 24 h after inoculation with powdery mildew fungus. To get the full-length cDNA, sequencing was performed by using T3 forward primers through an ABI 377 sequencer. As all of the cDNAs contained 3' polyA tail, cDNAs had 5' ATG that aligned with the start codons of genes known to encode homologues of the gene of interest were assumed to be full-length. Nucleotide sequences were analyzed by comparison with the databases using Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/blastx). The deduced amino acid sequence of the cDNA was analyzed by the program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted protein localization was obtained from (http://psort.nibb.ac.jp/form.html) and

(http://www.cbs.dtu.dk/services/TargetP). Multiple sequence alignment was performed by the program (http://searchlauncher.bcm.tmc.edu). A phylogenetic tree was constructed based on ClustalW 1.81 alignment (http://clustalw.genome.jp). Sequence analysis showed that MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605, SAMDC, and THFC/THFD might be localized in cytosol; SHMT might be localized in mitochondria.

My results indicated that in the compatible host interaction, expression of *MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605 and SAMDC* was synchronously up-regulated by powdery mildew infection and that all five genes have two expression peaks at 6 and 24 hpi. Peaks at 6 hpi are consistent with fungal primary germ tube penetration and peaks at 24 hpi are correlated with the appressorial germ tube penetration. In the incompatible interaction, the transcripts of all the five genes are coordinately induced by powdery mildew infection and reach a maximum at 24 hpi, which follows the time of formation of a large papilla underneath the appressorial germ tube penetration peg.

My results also showed that there are differences between responses provoked by the invasion of pathogenic organism and those provoked by abiotic stresses and stress signal molecules. Expression of *MTHFR, Met Syn, AdoMet Syn2661, AdoMet Syn605 and SAMDC* has much more variation in response to abiotic stresses and stress signal molecules. The differential expression patterns reflect the fine-tuning regulation of each gene under varied stimuli so that plants can avoid inappropriate gene expression. This can be evidenced by recent findings that there exist significant cross-talks among the signaling pathways (Cheong et al., 2002).

My results demonstrated that expression of *SHMT and THFC/THFD* was either constitutive or down-regulated in response to both biotic and abiotic stresses, which is different from *Tm-MTHFR*, *Tm-Met Syn*, *Tm-AdoMet Sy2661*, *Tm-AdoMeet Syn605* and *Tm-SAMDC*. These observations might be explained by the fact that both *SHMT* and *THFC/THFD* are involved in pathways crucial to many central activities in cells, such as serine biosynthesis for the generation of C-1 units, thymidylate and purine biosynthesis in general, and are, therefore, not linked specifically only to responses to stresses.

These results suggest a close metabolic link between stresses and the pathways of generation and supply of methyl units in this wheat.

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